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ORIGINAL ARTICLE

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Characterization of early aldosterone-induced RNAs identified in A6 kidney epithelia

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Abstract The early aldosterone-induced increase in Na reabsorption across tight epithelia is characterized by a transcription-dependent activation of epithelial Na channels (ENaC) and pumps (Na,K-ATPase). In order to contribute towards the identification of transcriptionally regulated mediators of this process, we first tested mRNAs of proteins previously suggested to be involved. Epithelia were treated for 1 h with 10^{-6} M aldosterone, a concentration which produces a maximal transport response and at which both mineralo- and glucocorticoid receptors are occupied. Northern blot analysis showed no change in mRNAs of trimeric G protein alpha subunits, calmodulin, and mitochondrial energy metabolism proteins, whereas Na,K-ATPase α1 and β1 subunit mRNAs were slightly increased (1.2- to 1.4-fold). In a second approach, we visualized 5000 cDNA bands generated from A6 RNAs by differential display polymerase chain reaction (PCR). After 1 h of aldosterone treatment, ≈0.5% of these appeared to be regulated. Four cDNA fragments corresponding to early adrenal-steroid-upregulated RNAs (ASURs) were cloned and for two of them cDNAs containing entire coding sequences were isolated by library screening. ASUR4 is the *Xenopus laevis* homologue of human E16 and rat TA1, a membrane protein structurally related to yeast and prokaryotic permeases, and ASUR5 is the A transcript of *Xenopus* K-*ras*2. The rapid inductions of the four ASURs correspond to direct transcriptional effects since they were not inhibited by cycloheximide but were blocked by actinomycin D. The $K_{1/2}$ values were similar or slightly below those reported for stimulation of Na transport. These characteristics of RNA accumulation and their time courses suggest a possible role of one of these induced RNAs in the mediation of the early effect of aldosterone on Na transport.

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Introduction

Aldosterone controls Na excretion by regulating its reabsorption across tight epithelia such as the distal nephron. In amphibian model epithelia, a maximal stimulation of Na reabsorption is obtained with aldosterone concentrations at which mineralo- and glucocorticoid receptors are occupied. This response can be divided into two phases (for review see [18, 34, 42, 45]). The first (early) phase starts after a lag period of ≈45 min and is characterized by an activation of apparently pre-existing epithelial Na channels [21] and Na pumps [7]. The second (late) phase starts ≈3 h after aldosterone addition and is characterized by an accumulation of structural elements of the Na transport machinery.

In some cases, the late aldosterone-induced accumulation of Na transport proteins results from an increase in the rate of transcription of corresponding genes. For instance, the delayed accumulation of functional Na pumps (Na,K-ATPase) in *Xenopus laevis* A6 epithelia has been shown to be preceded by a rapid activation of the Na,K-ATPase subunit genes [7, 44]. In the case of the epithelial Na channel (ENaC), a late accumulation of its β and γ subunit mRNAs has been observed in rat distal colon in response to aldosterone or a low-salt diet [4, 32].

The early aldosterone-induced activation of Na channels and Na pumps, which precedes the accumulation of new transport proteins, is also dependent on ongoing transcription and translation. Hence, it has been postulated that (an) early aldosterone-induced protein(s) would directly or indirectly act at the level of ENaCs and Na pumps [18, 42]. Different proteins have been suggested, based on functional and/or biochemical studies, to play a role in this action; for instance, a component of the ENaC [9], G protein α_{i3} [33], calmodulin [15] and oxidative phosphorylation enzymes [17, 30]. However, as yet no mRNA has been identified that has been shown to be

increased (or repressed) early enough to encode a protein possibly accounting for (part of) the early physiological effect of aldosterone.

In this study, we report that the mRNAs of the candidate mediators mentioned here are not regulated at all or not early enough to account for the early physiological response to aldosterone in A6 epithelia. In contrast, using differential display polymerase chain reaction (PCR), we have analysed the impact of short-term aldosterone treatments on the RNA population of A6 epithelia and cloned four cDNA fragments corresponding to the first early adrenal steroid-upregulated RNAs (ASURs) identified in a tight epithelium. Two ASURs do not correspond to known sequences and, for the two others, cDNAs which contain the entire coding region were obtained and sequenced. These new sequences represent the *Xenopus laevis* homologues of mammalian E16/TA1 and K-*ras*2A. Time course, dose/response relationships and the use of transcription and translation inhibitors show that the induction is, for all four ASURs, a direct transcriptional effect and suggests a mediation by the glucocorticoid receptor.

Materials and methods

Cell culture

A6 cells from the A6-C1 subclone were cultured on permeable supports as described previously [7]. RNA was prepared from epithelia cultured for a total of 15–18 days, 10 days in bicarbonate-buffered medium supplemented with 10% fetal calf serum and then in serum-free HEPES-buffered medium. Aldosterone or vehicle (ethanol 0.01%) was added to a final concentration of 10^{-6} M to both compartments when indicated. For dose/response experiments, concentrations of aldosterone of 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M were given for 2 h. The inhibitors actinomycin D (5 μg/ml) and cycloheximide (20 μg/ml, a concentration which has previously been shown to block 97% of the protein synthesis in A6 epithelia [44]) were given 5 min prior to aldosterone. Control experiments showed that the aldosterone treatment increased Na transport two- to five-fold within 3 h, as reported previously [6].

Differential display

Total cellular RNA was isolated with Trizol Reagent (Gibco) according to the manufacturer's protocol directly from filter-cultured A6 epithelia treated for 40 min or 1 h with aldosterone or vehicle. This RNA (50 μg) was treated with DNaseI (10 U) (Pharmacia) for 30 min at 37°C in 10 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, 100 mM KCl, 1 mM dithiothreitol (DTT) and RNasin (10 U) (Promega), extracted three times with phenol/chloroform/isoamyl-alcohol and precipitated with ethanol. Differential display PCR was performed according to the procedure published by Liang and Pardee [23] with some modifications and using following oligo(dT) and arbitrary primers ten nucleotides in length (10mer): T1 (T_{11}CA), T2c (T_{11}AAN), T3 (T_{11}AT), T4 (T_{11}CT), T5 (T_{11}AC), T6 (T_{11}GG), T7 (T_{11}GC), T8 (T_{11}AG), T9 (T_{11}GT), T10 (T_{11}CC), T11 (T_{11}CG), T12 (T_{11}GA) and O1 (GAAGCATCAC), O2 (GATGAAGTCC), O3 (ACATGCTAGG), O4 (ATCAACGTGC), O5 (GAGATTGGGA), O6 (CGTTACTAGG), O7 (GCATCAGATG), O8 (GACACGCTAT), O9 (CGGAGGAATA), O10 (CTTGATTGCC) (made by Microsynth, Balgach, Switzerland). Aliquots of 0.1 μg or 1.5 μg (for primer T1, T5, T6, T7, T8, T9, T10, T11 or T2c, T3, T4, T12, respectively) of RNA were denatured at 65°C for 10 min in the presence of 50 U RNasin (Promega) and 2×10^{-3} M DTT and used as templates for reverse transcription in a volume of 40 μl using BufferII (Perkin Elmer) with 2.5 μM oligo(dT) primer, 50 μM dNTPs, 5 mM $MgCl_2$ and 400 U MMLV reverse transcriptase (Gibco) for 1 h at 37°C. After heat inactivation (5 min at 95°C), 2 μl of the cDNA sample was used as a template for a 20-μl PCR reaction in BufferII with 2.5 μM oligo(dT) primer, 500 nM 10mer, 1.25 mM $MgCl_2$, 30 nM [α-^{32}P]dCTP (3000 Ci/mmol or 111 TBq/mmol) and 1.25 U Amplitaq DNA polymerase (Perkin Elmer). The cycling parameters were as follows: 94°C for 1 min as an initial denaturation step, 94°C for 45 s, 40°C for 3 min, 72°C for 1.5 min for 40 cycles followed by 72°C for 10 min.

Aliquots of the PCR reactions (1.5 μl or 5 μl for analytical or preparative gels, respectively) were separated on non-denaturing 6% acrylamide gels. After drying on Whatman 3MM paper and autoradiography, cDNA bands of interest were cut out. Gel slices were rehydrated in 200 μl H_2O for 10 min, boiled for 15 min and the DNA precipitated. Half of that DNA was used for reamplification with the same primer set and PCR conditions as above, except that 40 μM dNTPs and no tracer were used.

Cloning of reamplified cDNA fragments and sequencing

Reamplified cDNA fragments, which in some cases were smaller (probably due to internal priming) than the original PCR product used as the template, were eluted from agarose gels, treated with T4 DNA polymerase and T4 polynucleotide kinase (New England Biolabs) and ligated into pBluescript II (Stratagene). Plasmid DNA sequencing was made using the T7 Sequencing Kit (Pharmacia) with T3 and T7 primers and internal primers when required. The original ASUR fragments and all coding sequences were sequenced on both strands. Sequence similarities to known DNA sequences were searched with the basic local alignment search tool (BLAST, [1]) using nr, dbest, dbsts, yeast and mito programs of the National Center for Biotechnology Information (Bethesda, Md., USA).

Northern blot analysis

Total cellular RNA was isolated either with Trizol Reagent (Gibco) or following the original Chomczynski and Sacchi [13] procedure. Polyadenylated RNA was prepared by the Proteinase K method [20] with following modifications: filter-cultured A6 cells were directly scraped in proteinase K solution [20 mM Tris (pH 7.5), 1 mM EDTA, 0.1 M NaCl, 0.5% SDS and 200 μg/ml Proteinase K (Merck)] and homogenized with a polytron PT10-35 (Kinematica). Poly(A) RNA was selected on oligo(dT) cellulose (Gibco). Total RNA (5 μg) or poly(A) RNA (1.5 μg) and RNA standards (Promega) were run on 1% agarose/formaldehyde gels, transferred to Genescreen membranes (NEN Dupont) and immobilized with ultraviolet light according to standard protocols. Probes labelled with [α-^{32}P]dCTP to a specific activity of 0.5×10^9 to 2×10^9 cpm/μg DNA were generated by random priming (Oligolabelling kit, Pharmacia). Hybridization and washes were performed according to standard protocols. Blots were exposed, scanned and signals quantified using a Phosphorimager and the Imagequant software (Molecular Dynamics). Mean fractional changes of the signal intensity (relative to the actin control for the "candidate" blots) ± standard error are indicated for *n* blots made with RNA prepared from different experiments. The effect of inhibitors (cycloheximide and actinomycin D) was tested on two blots from different experiments. For determinations of the dose/response relationship, the means of the signals from three or four blots (made with different RNA preparations) were used to fit sigmoidal dose/response curves with variable slopes using the Prism software (Graphpad) (R^2 between 0.92 and 0.98).

Library screening

A cDNA library of filter-cultured A6 cells treated for 4 h with 10^{-6} M aldosterone was made with the Superscript system (Gibco)

from total poly(A) RNA prepared as above, and another library of filter-cultured A6 cells treated for 24 h with 3×10^{-7} M aldosterone made from size-fractionated poly(A) RNA was a gift of A. Puoti [29]. Sublibraries were tested by PCR with ASUR-specific primer combinations. Positive sublibraries were then screened by colony hybridization with labelled ASUR-cDNA.

Results

Effect of aldosterone on mRNAs of candidate mediators and Na pump in A6 epithelia

The early effect of aldosterone on the mRNA of different candidate mediators of the aldosterone action was tested on Northern blots made with RNA prepared from filter-cultured A6 cells using *Xenopus laevis*-specific probes (Fig. 1). The tested mRNAs corresponded to trimeric G protein α subunits, calmodulin, mitochondrial transcription factor A (mtTFA) and cytochrome *c* oxydase subunit I. Actin (*X. laevis* cytoskeletal actin type 8 [27]) was used as negative control and Na,K-ATPase α1 and β1 subunits as positive controls for a late aldosterone effect [44]. It should be mentioned that both mineralocorticoid

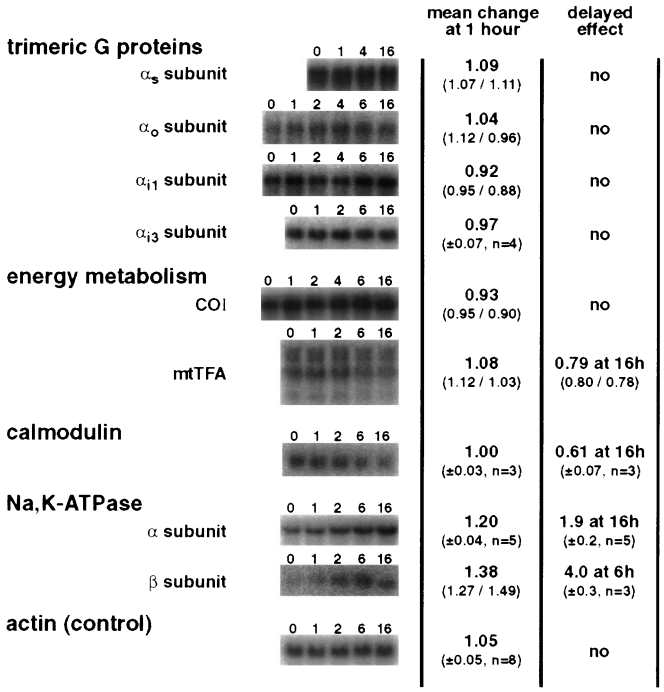

Fig. 1 Effect of aldosterone on mRNAs of candidate mediators for the aldosterone action and Na pump (Na,K-ATPase). Times of aldosterone treatment (hours) are indicated for each Northern blot. The signals were normalized to those of actin (with the exception of actin itself) and the fractional changes (aldosterone/control) are indicated. Means of two blots made with RNA from different experiments (both values indicated) or more than two blots (± standard error, *n* = number of experiments) are given. Following *Xenopus* (c)DNAs were used as probes: trimeric G protein α subunit cDNAs [28], cytochrome *c* oxidase subunit I (*COI*) from mitochondrial DNA [31], nuclear encoded mitochondrial transcription factor A cDNA (*mtTFA*) [3], calmodulin cDNA [12], Na,K-ATPase cDNAs [43] and type 8 actin [27]

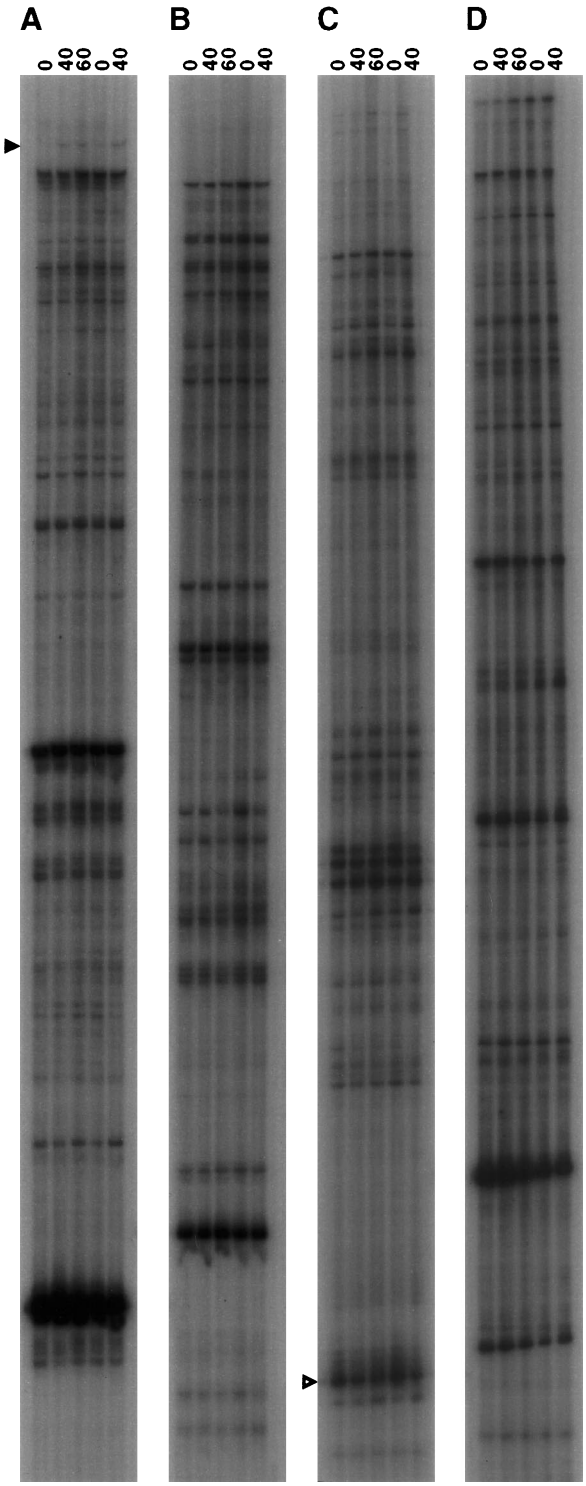

Fig. 2A–D Differential display from control and aldosterone-treated A6 epithelia using five different RNA samples from two different experiments: section of an autoradiogram. Times (min) of aldosterone treatment (10^{-6} M) are indicated. Primer T2c was used as anchored oligo(dT) primer in combination with primer O2, O3, O8 and O9 (**A, B, C** and **D**, respectively). Bands showing altered expression for all three test RNAs are marked by *open* or *filled arrowhead* for increasing or decreasing signals, respectively

Table 1 Early adrenal-steroid-upregulated RNAs (*ASURs*): characterization of cDNA fragments and corresponding RNAs

	ASUR1	ASUR2	ASUR4	ASUR5
Length of cloned cDNA fragment (bp)	463	356	244	570
RNA on Northern blots: approximate length (kb)	8.5 and 11	2.5–10.5	5.1	2.6
RNA on Northern blots – fractional change (mean±SEM of n=3–5 blots) after:				
1 h 10^{-6} M aldosterone	4.1±0.6	15.2±2.4	1.7±0.3	2.7±0.4
2 h 10^{-6} M aldosterone	4.8±0.7	14.7±2.0	1.8±0.2	4.4±0.5
RNA on Northern blots: induction in presence of cycloheximide	yes	yes	yes	yes
RNA on Northern blots: induction in presence of actinomycin D	no	no	no	no
RNA on Northern blots: $K_{1/2}$ for aldosterone effect (M)[a]	4.1×10^{-9}	5.1×10^{-9}	2.4×10^{-8}	3.2×10^{-8}
Mammalian homologue identified after "full-length" cDNA cloning	n.d.	n.d.	E16/TA1	K-*ras*2A
EMBL Data Library accession number	Y12717	Y12718	Y12716[b]	Y12715[b]

[a] The values were obtained by fitting sigmoidal curves to the data points (see Fig. 3C)
[b] These accession numbers are for the "full length" cDNA sequences

and glucocorticoid receptors are expected to be activated [14, 39] with the concentration of aldosterone used (10^{-6} M). This concentration was chosen because it is known to give a maximal early physiological response in the A6-C1 system. The progressive increase in Na,K-ATPase mRNA confirms earlier observations and shows that the response to aldosterone is similar in the present culture conditions using the A6-C1 clone as compared with earlier conditions and the A6-2F3 clone [44]. We have previously reported an increase in Na,K-ATPase at the protein level (total and cell surface) which started to be significant 5 h after aldosterone addition [6]. Cytoskeletal actin type 8 is an adequate control since it was not modified by aldosterone. None of the tested mRNAs was regulated within 1 h of aldosterone treatment. At later time points, calmodulin and mtTFA transcripts were slightly downregulated while the other candidates were not significantly affected.

Effect of short-term aldosterone treatment on the RNA population of A6 epithelia visualized by differential display

We performed differential display PCR [23] using RNAs from filter-cultured A6 epithelia, either treated for a short period with 10^{-6} M aldosterone [40 min (n = 2) or 1 h (n = 1)] or maintained under control conditions (n = 2). We visualized approximately 5000 cDNA fragments generated with 120 different primer pairs composed of an anchored oligo(dT) and a random 10mer primer (Fig. 2). Using arbitrary criteria (parallel change in all test RNAs by ≥ 1.5-fold), we counted 31 (≈0.6%) upregulated cDNA fragments and 5 (0.1%) downregulated ones.

Cloning of four cDNA fragments corresponding to early ASURs

We reamplified and cloned cDNA fragments corresponding to 15 regulated bands. Frequently, more than a single type of cDNA fragment was cloned from a single band. A total of 52 fragments were tested on Northern blots made from poly(A) and total RNA of control and aldosterone-treated A6 epithelia. In four cases of upregulated and none of downregulated cDNA bands, one of the cloned fragments was shown to correspond to an early aldosterone-regulated RNA species (Table 1). The other cDNA fragments corresponded either to RNAs that were not regulated, or to RNAs that were not abundant enough to produce a signal on Northern blots. We named the upregulated cDNAs ASUR for aldosterone- or adrenal steroid-upregulated RNA. The induction of all four ASURs was prevented by the transcriptional inhibitor actinomycin D, but not inhibited by the translation inhibitor cycloheximide, indicating that it was a direct effect at the transcriptional level. Dose/response experiments showed that half-maximal upregulations ($K_{1/2}$) were obtained with 4–32 nM aldosterone (Table 1 and Fig. 3C).

ASUR1 produced two signals on Northern blots at approximately 8.5 and 11 kb which were rapidly upregulated by aldosterone and remained high up to 16 h of treatment (≈fivefold increase) (Table 1 and Fig. 3A, B). Both bands were enriched by oligo(dT) selection but there was nearly no signal on blots made with cytoplasmic poly(A) RNA (data not shown). This suggests that these RNAs could be nuclear and could represent mRNA precursors. In this case, the cloned fragment would be intronic or the abundance of the mature mRNA would be very low. Alternatively, the nuclear RNA might have an as yet unknown function. Using BLAST (basic local alignment search tool), we could not find any significantly similar sequence in the databases.

The ASUR2 cDNA produced on Northern blots a reproducible pattern containing several bands which was acutely and massively increased by aldosterone (15-fold after 1 h, measuring the signal intensity over the entire smeary pattern) (Table 1 and Fig. 3A, B). This effect was only transient, since the signal started to progressively decrease at 2 h of treatment. Interestingly, the hybridizing RNA species was not enriched but decreased by oligo(dT) sepharose chromatography, indicating that it did not correspond to a polyadenylated RNA. The complex pattern produced on Northern blots suggests that the ASUR2 cDNA hybridizes to products of a family of

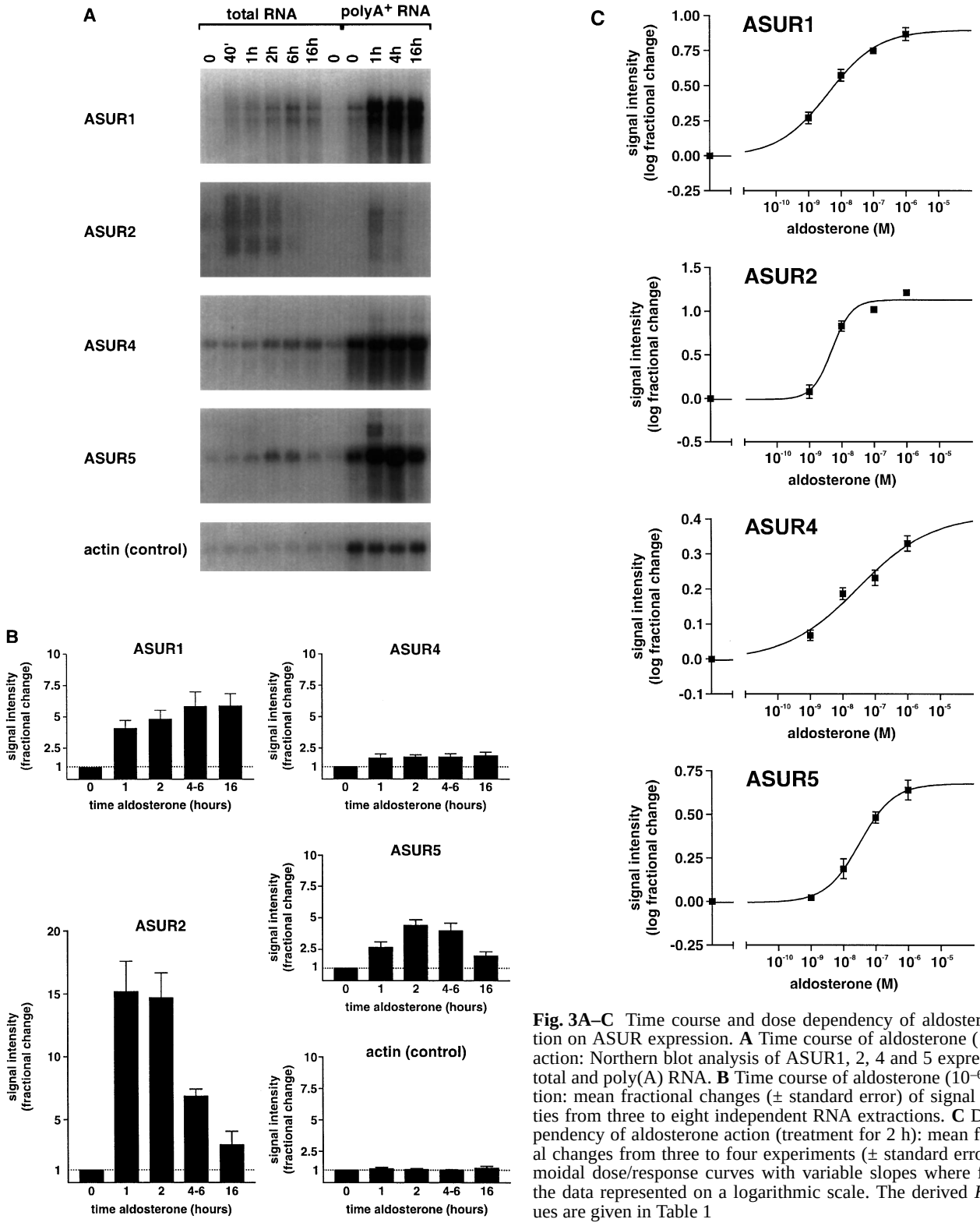

Fig. 3A–C Time course and dose dependency of aldosterone action on ASUR expression. **A** Time course of aldosterone (10^{-6} M) action: Northern blot analysis of ASUR1, 2, 4 and 5 expression in total and poly(A) RNA. **B** Time course of aldosterone (10^{-6} M) action: mean fractional changes (± standard error) of signal intensities from three to eight independent RNA extractions. **C** Dose dependency of aldosterone action (treatment for 2 h): mean fractional changes from three to four experiments (± standard error). Sigmoidal dose/response curves with variable slopes where fitted to the data represented on a logarithmic scale. The derived $K_{1/2}$ values are given in Table 1

highly conserved and coregulated genes or to differentially spliced products from a single gene. Using BLAST, we could not find in the databases any sequence with significant similarity to ASUR2.

ASUR4 is the *X. laevis* homologue of mammalian E16/TA1

The signal produced by the ASUR4 fragment on Northern blots indicated that it corresponded to a RNA of ≈5 kb which was upregulated 1.7-fold within 1 h by al-

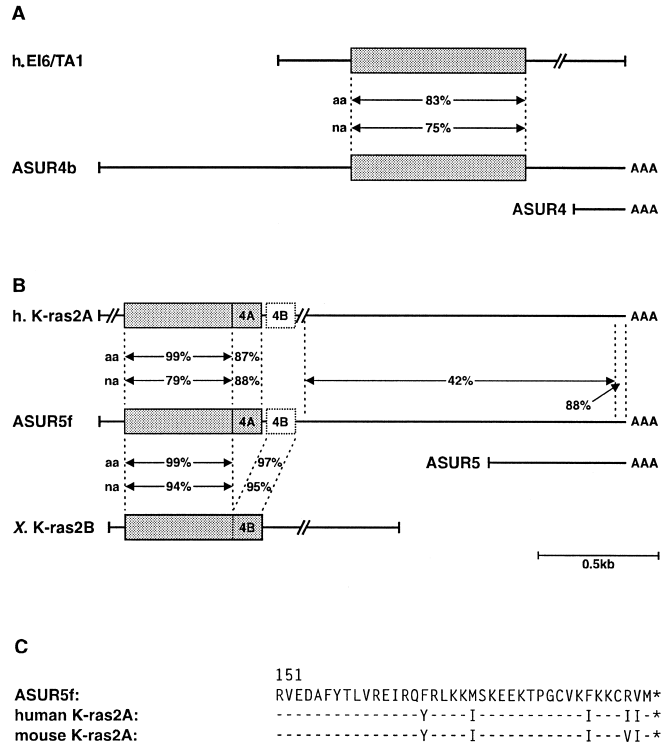

Fig. 4A–C Schematic drawing of ASUR4 and ASUR5 and sequence comparison with human homologues. Coding sequences are represented as *dark boxes*, untranslated open reading frames (*ORFs*) as *open boxes*. **A** Comparison of ASUR4b with human E16 (GenBank M80244) on nucleotide (*na*) and amino acid (*aa*) levels. **B** Comparison of ASUR5f with the 4A transcript of human K-*ras*2 (GenBank P01116). Note that the degree of identity is smaller at the level of the variable C-terminus than for the rest of the protein. The comparison of ASUR5f with *Xenopus* K-*ras*2B (GenBank X53246) shows at the nucleotide level 94% identity for the part of the ORF encoded by exon 1–3 and 95% for the C-terminal part which is encoded by exon 4B (this sequence is part of the 3′ UTR in the 2A type transcripts). **C** Alignment of the amino acid sequence encoded by exon 4A of human K-*ras*2A, mouse K-*ras*2A (GenBank P32883) and ASUR5f. Unidentical amino acids are indicated

dosterone (Table 1 and Fig. 3A, B). Using the ASUR4 cDNA fragment as a probe, we isolated a 2.24 kb cDNA (ASUR4b) from a library made from filter-cultured A6 cells treated with 10^{-6} M aldosterone for 4 h. Interestingly, both the original cDNA fragment and the longer cDNA appeared to have been primed from a poly(A) stretch that is not preceded by a polyadenylation signal and which might be part of a long 3′-untranslated region. The new cDNA contains a coding sequence of 723 bp, 1050 bp of 5′UTR and 457 bp of 3′UTR plus a stretch of 15 A's. The translation of the open reading frame yielded a product which is 83% identical with human E16 [19] and 82% with rat TA1 [35] (Fig. 4A). Northern blots hybridized with a probe made from the coding sequence gave the same result than those made with the original ASUR4.

ASUR5 is the *X. laevis* homologue of mammalian K-*ras*2A

Northern blots hybridized with ASUR5 cDNA fragment showed a signal at 2.6 kb which was clearly increased by aldosterone (2.7-fold after 1 h, 4.4-fold after 2 h) (Table 1 and Fig. 3A, B). After stimulation with aldosterone, an additional weaker signal for a longer RNA species, possibly a precursor, appeared. The main signal decreased to some extent but remained above control at 16 h of aldosterone treatment. Using the ASUR5 fragment as the probe, we cloned three independent clones which all contain an open reading frame for the A splice variant of a *X. laevis* K-Ras2 protein from a library produced with RNA from filter-cultured A6 cells treated for 24 h with aldosterone (3×10^{-7} M). The largest clone (ASUR5g) contains 119 bp 5′UTR. Clone ASUR5f was entirely sequenced and contains 101 bp 5′UTR, a 567 bp open reading frame encoding a protein of 189 amino acids, and a 1489 bp 3′UTR plus a poly(A) tail. The 3′UTR contains the 4B exon which starts, as determined for the human K-*ras*2 gene [26], five bases after the stop codon of the K-*ras*2A coding sequence. This sequence encodes the last 37 amino acids of the K-Ras2B form (Fig. 4B). The degree of identity to the human homologue is indicated in Fig. 4B. It is interesting to note that both species share a nearly identical stretch of 3′UTR just before and including the polyadenylation signal (30 from 34 nucleotides identical). Northern blots hybridized with a probe produced with a K-*ras*2A specific fragment generated by PCR (*X. laevis* DNA fragment corresponding to the human 4A exon, see Fig. 4B) yielded the same band and regulatory pattern as with the original ASUR5 cDNA, confirming that the K-*ras*2A variant is upregulated by aldosterone in A6 epithelia.

Discussion

The aim of this study was to identify those mRNAs of A6 epithelia which are regulated by aldosterone during the lag period of its early physiological action. The choice of the early time points is based on the assumption that the increase in Na reabsorption, which is dependent on ongoing transcription and translation and inhibitable by corticosteroid receptor antagonists, is mediated (directly or indirectly) by aldosterone-induced/repressed regulatory proteins acting at the level of the pre-existing Na transport machinery, in particular at the level of the Na channels and Na pumps [18, 34, 42].

Candidate approach

Recently it has been suggested that aldosterone regulates Na channel activity in A6 cells via stimulation of expression and targeting of the α_{i3} subunit of a heterotrimeric G protein [33]. We have tested this hypothesis at the mRNA level using *X. laevis* probes for four trimeric G

protein α subunits, including the $G\alpha_{i3}$. None of the corresponding mRNAs was modified significantly within 1 h, excluding the possibility that a transcriptional regulation of G protein α_{i3} or of another of the tested α subunits mediates the early aldosterone response in our A6-C1 system.

Based on physiological studies on amphibian early distal tubule, Cooper and Hunter [15] proposed that calmodulin could be the induced mediator of the early aldosterone effect, which in this nephron segment is characterized by the activation of Na/H exchange. In A6 cells, we observed no significant change of the calmodulin mRNA after 1 h of aldosterone treatment. Interestingly, at later time points there was a slight decrease of this mRNA. This result clearly shows that calmodulin is not a transcriptionally regulated mediator of the early aldosterone effect in A6 cells. However, it is not excluded that this gene product plays an important role in the response to aldosterone in other nephron segments.

The supply of ATP by the mitochondria has been proposed to play a role in the control of Na transport by aldosterone. In particular, earlier studies have demonstrated that several mitochondrial enzymes were regulated by aldosterone in toad bladder and mammalian target epithelia [22, 24]. Using a differential screening approach to search for dexamethasone-induced mRNAs in rat distal colon, Rachamim et al. [30] recently identified mitochondria-encoded subunits of cytochrome *c* oxidase. The mechanism by which the mitochondrial RNA is induced by corticosteroid hormones is not known. In the present study, we tested whether the RNA of a mitochondria-encoded cytochrome *c* oxidase subunit and of the nuclear encoded mitochondrial transcription factor A [3], as a potential mediator of a steroid action on mitochondrial transcription, were regulated by aldosterone in A6 cells. Interestingly, these RNAs were not upregulated, neither early nor late, suggesting that, in A6 cells, aldosterone does not act at the level of mitochondrial ATP supply. It has been previously shown that citrate synthase is not induced by aldosterone in A6 cells [41].

It has been suggested, based on protein two-dimensional gel analysis and antibody work, that a component of the ENaC is induced in its synthesis rapidly enough to possibly account for the early aldosterone effect [9]. As yet three homologous subunits of this channel (ENaC) have been cloned [10, 29], but it is not established whether one of these corresponds to the induced protein mentioned above. In any case, May et al. [25] have shown in a preliminary report that none of the three *x*ENaC mRNAs is regulated in A6 epithelia early enough to account for the early physiological effect. Furthermore we have measured by Northern blotting the regulation of the three *x*ENaC subunit mRNAs in our A6-C1 system. At an early time point (1 h), only the γ subunit mRNA was slightly increased similarly to the Na,K-ATPase subunits (1.31 ± 0.06, $n = 3$). All three subunit mRNAs were increased approximately twofold after 16 h (B. Spindler and F. Verrey, unpublished observation). Hence, either the induced protein described by Blazer-Yost is not a known channel subunit or, alternatively, the regulation could take place at a translational or post-translational level. In such a case, one would have to postulate the presence of another transcriptionally induced/repressed regulatory protein (or RNA) to account for the translational or post-translational regulation.

Other hypotheses such as those proposing as mediator a methylase [37] or (a) G protein(s) not belonging to the tested trimeric G proteins [38] could not be tested since these candidates are biochemically not defined.

Differential display PCR

Differential display PCR is a technique with the appealing potential to visualize the differences and similarities between two RNA populations, analogous to two-dimensional gel analysis at the protein level, and, furthermore, to allow the cloning of cDNA fragments of interest [23]. However, since it is based on the consecutive use of reverse transcription and PCR (in low-stringency conditions) it also bears the risk of producing artefacts. To decrease the risk of identifying false positives, we performed the reactions on RNAs from two independent extractions using two control and three test RNAs. The numbers of regulated fragments indicated in the Results have nevertheless to be taken with caution. Indeed, changes as small as ≈1.5-fold were accepted. It is likely that the proportion of acutely and substantially regulated RNAs is actually much smaller than the indicated 0.6% (upregulated) and 0.1% (downregulated). This is also suggested by the fact that in a number of cases the cDNA fragments cloned from apparently regulated bands were not confirmed as corresponding to regulated RNAs by Northern blotting. In any case, it is relevant to know that clearly less than 1% of the expressed RNAs appears to be acutely regulated by aldosterone in A6 epithelia. The mRNA(s) encoding the putative regulator(s) of the Na channels and Na pumps are expected to be among this relatively small number of regulated RNAs.

We have cloned four cDNA fragments which correspond to early aldosterone-upregulated RNAs in A6 epithelia. Using inhibitors of transcription and translation we showed that the effect of aldosterone is transcriptional and direct in all four cases. The dose dependency of the RNA upregulations suggests mediation by the glucocorticoid receptor. Indeed, the K_d of aldosterone for the A6 cell gluco- and mineralocorticoid receptors was estimated to 39 and 1.25 nM, respectively, in bindings studies conducted at 0°C [14]. Furthermore, the activation of Na transport has been shown to be mediated in A6 epithelia by the glucocorticoid receptor with a $K_{1/2}$ estimated to ≈50 nM aldosterone [39]. However, in view of the fact that a substantial part of the upregulation of ASUR1, 2 and 4 takes place at concentrations of aldosterone in the low nanomolar range, a role of the mineralocorticoid receptor is not excluded (Fig. 3C). Interestingly, ASUR1 and ASUR2, which are highly regulated, are not typical mRNAs, and no significant simi-

larity to published sequences has been identified using BLAST (see Results).

In contrast, the ASUR4 and ASUR5 fragments have been used successfully to obtain cDNAs containing the entire coding sequence from *X. laevis* homologues of known proteins. ASUR4 corresponds to human E16 (transiently expressed mRNA in activated lymphocytes [19]) and rat TA1 (expressed in hepatoma cells [35]) (Fig. 4A). The encoded protein of ≈27 kDa is very hydrophobic and resembles prokaryotic amino acid transporters [47], but its function is as yet unknown. Based on its similarity to permeases and because of the relatively low amplitude of its regulation by aldosterone, it does not appear to be a prime candidate for the regulation of Na transport. One possibility is that this protein mediates the transport of (a) metabolite(s) which might or not play a role in the support of Na transport.

From an A6 cell library, we have cloned three independent cDNAs for ASUR5 which all correspond to the A splice variant of K-*ras*2 [26, 40] (Fig. 4B). This form of K-*ras*2 differs from the more common K-*ras*2B at the level of the fourth exon which encodes the "variable" C-terminus of $p21^{ras}$ known to be involved in its association with membranes [16, 46]. In contrast to the B form of K-Ras2, which has a lysine string required for membrane binding, the A form has a potential palmitoylation site. As yet two different K-*ras* cDNAs had been cloned from *X. laevis* oocyte libraries which both correspond to K-*ras*2B forms [2, 5]. The newly isolated cDNA is most similar to that published by Baum and Bebernitz [5] and differs from it in the common part of the coding sequence by 1% at the amino acid level and 6% at the nucleotide level. This extent of divergence is similar to that seen for other *X. laevis* genes which were duplicated approximately 30 million years ago [8]. Hence, it is likely that these two *X. laevis* K-*ras*2 cDNAs are derived from such non-allelic homologous genes. The degree of identity of the newly cloned *X. laevis* K-*ras*2A cDNA and its human homologue is 97% at the amino acid level (Fig. 4B). The comparison of the exon 4A encoded C-terminal regions is shown in Fig. 4C. In view of the fact that K-*ras*2A has been shown to be by far the least expressed splice variant of K-*ras*2 in human tissues and cells [11], and that a role has been postulated for G proteins other than trimeric G proteins in the mediation of Na channel regulation by aldosterone [36, 38], it will be interesting to study whether there is a functional link between the induction of this K-*ras* and the early aldosterone-induced stimulation of Na channels and Na pumps.

In conclusion, only a small proportion of the RNAs expressed in A6 cells are regulated within the lag phase of the physiological action of aldosterone and hence potentially encode mediators of this action. We have cloned cDNAs corresponding to such early ASURs and characterized their induction. ASUR4 encodes a putative membrane protein related to permeases and ASUR5 K-Ras2A. It will be interesting to functionally test whether one of these ASURs participates in the mediation of the physiological response to aldosterone.

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