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REGULATION OF ASPARTATE AMINOTRANSFERASE MESSENGER RIBONUCLEIC ACID LEVEL BY TESTOSTERONE

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Summary—The effect of testosterone on precursor mitochondrial aspartate aminotransferase (pmAAT) mRNA was studied in rat ventral prostate and primary cell cultures of mini-pig prostate. Testosterone induced a 2–3-fold increase in pmAAT mRNA level in both rat ventral prostate and mini-pig prostate cultures. The pmAAT mRNA induction occurred 30 min after testosterone treatment and was maximal by 1.5 h. Prostatic mAAT activity was also induced by testosterone with a 1–2 h lag period. The time-course of induction of pmAAT mRNA, pmAAT activity and mAAT activity was consistent with stimulation of mRNA synthesis followed by increased synthesis and import of pmAAT into mitochondria. The effect of testosterone on pmAAT mRNA was specific because the increase in pmAAT mRNA was at least 2-fold greater than the increase in poly (A^+) RNA. These results suggest that testosterone stimulated mAAT activity by induction of pmAAT mRNA. This continues to support our proposal that a major physiological effect of testosterone is increased pmAAT mRNA steady-state levels which result in increased pmAAT synthesis and increased mAAT activity. These changes ultimately result in increased citrate production by prostate epithelial cells.

INTRODUCTION

A major physiological characteristic of the prostate in many mammals is citrate accumulation and secretion. In the rat, ventral prostate accumulates an extraordinarily high level of citrate as compared with other soft tissues. Citrate accumulates in rat ventral prostate as a result of limited citrate oxidation because of a limited aconitase activity [1]. Since citrate oxidation is limited, generation of oxalacetate (OAA) by normal Krebs cycle activity is also limited. Therefore, an extra-Krebs cycle source of 4 carbons for production of OAA and subsequent synthesis of citrate must be available. We proposed that a mitochondrial aspartate–glutamate–citrate pathway functions in prostate [2]. This pathway provides OAA for citrate synthesis via intramitochondrial transamination of aspartate which is catalyzed by mitochondrial aspartate aminotransferase (mAAT).

Testosterone regulates the level of citrate in rat ventral prostate. Ventral prostate citrate levels decrease after castration and are restored by testosterone administration [3]. Testosterone appears to mediate this effect on prostate citrate by increasing mAAT activity [4]. We previously showed that testosterone stimulated mAAT activity in ventral prostate but not in liver or kidney [4]. Moreover, testosterone stimulated mAAT activity as well as increased citrate production from aspartate in similar dose-dependent manners [5]. The increase in mAAT activity after testosterone treatment was the result of an increase in mAAT apoenzyme [6] and was blocked by cycloheximide and actinomycin D [7].

Generally, testosterone effects occur primarily via changes in the levels of mRNAs of testosterone stimulated proteins. The present study addresses the question of the effect of testosterone on the steady-state level of pmAAT mRNA. The *in situ* effects of testosterone on pmAAT mRNA were investigated using mature castrated rats. The time-course of the testosterone stimulated increase in pmAAT mRNA was determined using primary cultures of mini-pig prostate.

EXPERIMENTAL

Animals

Mature male Wistar rats (200–300 g) were used. Rats were obtained from Harlan Sprague–Dawley, Indianapolis, Ind. Animal preparation, castration, testosterone treatment and prostatic tissue preparation were performed as previously described [8].

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Abbreviations: Poly(A^+)RNA, poly-adenylated ribonuclei acid; pmAAT, precursor mitochondrial aspartate aminotransferase; mAAT, mitochondrial aspartate aminotransferase; cAAT cytosolic aspartate aminotransferase; PAGE, polyacrylamide gelelectrophoresis.

Preparation of polysomal RNA

Rats were killed by decapitation and the livers removed and washed in ice-cold H1 buffer (20 mM HEPES, 75 mM potassium chloride, 100 mM potassium acetate, 5 mM magnesium acetate, 1 mM dithiothreitol, pH 7.6). The livers were cut into small pieces and homogenized in 3 volumes of ice-cold H2 buffer (same as H1 buffer with 250 mM sucrose and 1 μg/ml RNase inhibitor). Free and membrane bound polysomes were isolated from the homogenate by sucrose gradient centrifugation and detergent treatment [9]. Polysomal RNA was recovered by phenol/chloroform extraction followed by ethanol precipitation.

Preparation and labeling of pmAAT DNA probe

A 24 mer oligodeoxynucleotide probe complementary to the mRNA encoding amino acids 272–280 of rat liver pmAAT was synthesized using phosphoramidite chemistry. The sequence was derived from the published sequence of the complete pmAAT cDNA [10]. This region of pmAAT was selected because of its low homology with cAAT [11]. The oligodeoxynucleotide was synthesized using a Cyclone DNA synthesizer (MilliGen/Biosearch). The probe was removed from the synthesis column and purified by PAGE according to the protocol provided by the MilliGen/Biosearch corporation. The probe was biotin labeled using a photobiotin labeling system purchased from Bethesda Research Laboratories (BRL). The effectiveness of the photolabeling reaction was determined by testing the biotin labeled DNA in a dot-spot assay using the BluGENE nonradioactive nucleic acid detection system from BRL.

Slot-blot hybridization

The relative amount of pmAAT mRNA in samples of poly(A^+)RNA was determined by slot-blot analysis. Aliquots of sample, containing 1.0 μg of RNA, were transferred to nitrocellulose filters using a Bio-Dot Slot Format Blotting apparatus (Bio-Rad Laboratories). The nitrocellulose filters were dried under a lamp and baked under vacuum at 80°C for 2 h. The nitrocellulose filters were prehybridized, hybridized and washed according to a slight modification of the method of Thomas[12]. The filters were soaked in 2 × SSC buffer (1 × SSC = 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0) and incubated in 0.1 ml/cm^2 of prehybridization buffer (50% formamide, 5 × SSC, 25 mM sodium phosphate, pH 6.5, 0.1% each Ficoll, polyvinylpyrrolidone, BSA and 0.5 mg/ml denatured sheared herring sperm DNA) for 4 h at 42°C. After prehybridization, the buffer was replaced with the same volume of hybridization buffer (45% formamide, 5 × SSC, 25 mM sodium phosphate, 0.01% each Ficoll, polyvinylpyrrolidone, BSA, and 0.2 mg/ml denatured sheared herring sperm DNA) containing 100 ng/ml biotinylated DNA probe. The amount of probe used in hybridization reactions was determined by comparing the newly biotinylated probe with a biotinylated standard using dot-spot assay. The blots were hybridized for 18–20 h at 55°C. After hybridization the filters were washed twice in 2 × SSC and 0.1% SDS for 3 min each at room temperature, twice in 0.2 × SSC and 0.1% SDS for 3 min each at room temperature and twice in 0.16 × SSC and 0.1% SDS at 50°C for 15 min each. Biotinylated probe bound to the RNA blots was detected using the BlueGENE detection system. The amount of bound probe was determined by densitometer scanning.

Preparation of animals and prostate tissue

Male Wistar rats (275–300 g) were castrated and 18–24 h later the animals were divided in two groups. One group was injected (subcutaneous) with 1 mg/animal testosterone propionate, the other group with the corn oil vehicle. The animals were fasted overnight and killed the next day. The prostates were removed, homogenized and free polysomes prepared as described above. Poly (A^+)RNA was isolated by a batch method using oligo (dT) [13]. The poly (A^+)RNA was blotted on to nitrocellulose filters and hybridized with the oligonucleotide probe as described above. The amount of pmAAT mRNA was determined by densitometer scan of the filters.

Cell culture

Primary cultures of mini-pig prostate cells were established as described previously [14]. The cells were cultured in Eagle's minimum essential medium with D-valine (MEM D-valine) supplemented with 10% FBS (Hyclone), 0.2 IU/ml insulin (Sigma), and 10^{-8} M testosterone (Sigma) in a humidified atmosphere of 95% air 5% CO_2. Cells were grown to 100% confluency, general within 7–10 days with medium changed every 2 days. For hormone treatment the medium was changed to serum free medium (SFRE 199-2, Sigma) containing insulin, and no testosterone on the day before the experiment. After incubation for 18–20 h in SFRE 199-2 without testosterone the cells were switched to SFRE 199-2 containing 10^{-8} M testosterone. After various times of testosterone treatment, the cells were collected in buffer using a rubber policeman. The cells were homogenized, and free polysomes prepared as described above. Mitochondria and cytosol were prepared, and mAAT and pmAAT activities were determined by the methods described previously [6]. Total poly (A^+)RNA was determined by slot-blot hybridization with a synthetic 25 mer poly (dT) probe.

RESULTS

Characterization of the oligodeoxynucleotide probe

We determined the specificity of the oligodeoxynucleotide probe by hybridizing the probe with increasing amounts of RNA isolated from rat liver free

and membrane bound polysomes. The amount of probe bound to the free polysome fraction increased as the amount of RNA increased (Fig. 1). However, the probe did not hybridize with RNA isolated from the membrane bound polysome fraction. Like most intracellular proteins pmAAT is synthesized on free polysomes in the cytosol and is not found associated with the membrane bound polysomal fraction [9]. Therefore, these results were consistent with the conclusion that the probe was specific for pmAAT mRNA. In addition, we performed hybridization reactions under stringent washing conditions and observed no binding of the probe to yeast RNA, nor to sheared, denatured calf thymus DNA (data not shown). However, the probe did hybridize to free polysomal RNA under the same conditions. Thus, these results taken together indicated that the probe was specific for pmAAT mRNA.

Effect of testosterone on pmAAT mRNA in rat ventral prostate

In order to determine the effect of testosterone on the level of pmAAT mRNA, we compared the amount pmAAT mRNA in cytosol extracts of ventral prostate from castrated, testosterone-treated, and sham animals. Rats were either castrated or sham operated by the procedure we have described [4]. After 24 h of testosterone treatment the animals were killed and the prostates removed. Poly (A^+)RNA was isolated from the free polysomal fraction, and pmAAT mRNA determined by slot-blot hybridization. Castration decreased the pmAAT mRNA level to 30% of sham in 48 h (Fig. 2). Testosterone treatment restored the pmAAT mRNA level to 90% of sham. The total free polysomal RNA decreased to 48% of the sham value after castration. These results were consistent with results reported for the effect of testosterone on mAAT activity [5, 6].

Fig. 1. Slot-blots of RNA isolated from cell fractions. Membrane bound polysomes (lane 1), nuclei (lane 2) and free polysomes (lane 3) prepared from rat liver. Rows a, b, and c represent 0.01, 0.1 and 1.0 μg repectively of RNA bounded to the nitrocellulose filter. The filter was hybridized with a 24 mer synthetic pmAAT mRNA oligodeoxynucleotide probe.

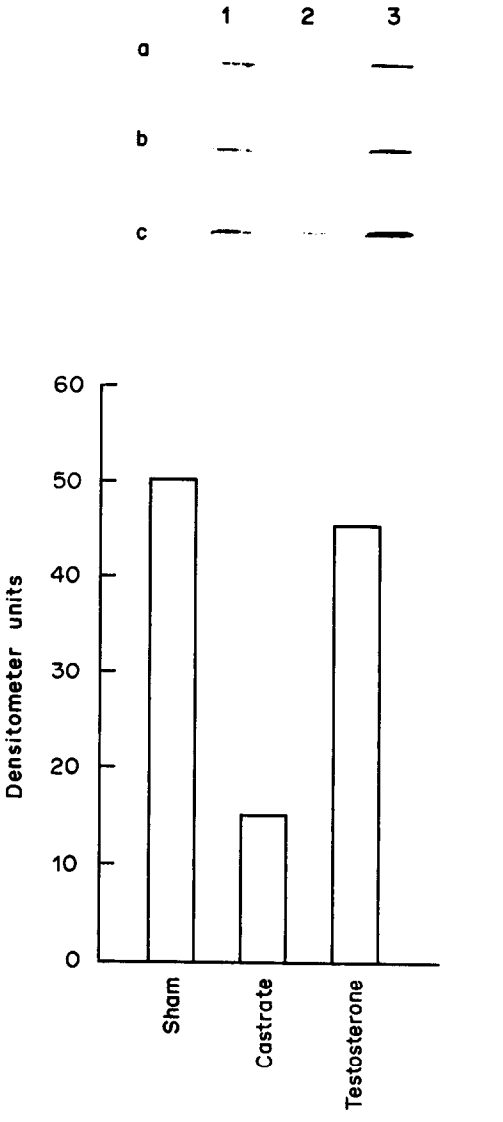

Fig. 2. The effect of castration and testosterone administration on pmAAT mRNA level of rat ventral prostate. After 18–20 h of castration rats were injected sucutaneously with 1 mg of testosterone propionate. 24 h later the animals were killed, the prostates removed and poly(A^+)RNA isolated from the free polysome fraction. Poly(A^+)RNA was blotted onto nitrocellulose filters and hybridized to a specific pmAAT mRNA probe. Lane 1 is castrate + testosterone, lane 2 is castrate + vehicle, and lane 3 is sham operated. Rows a, b, and c are 0.01, 0.1 and 1.0 μg of RNA, respectively. Graph shows results of densitometer scan of row c.

Time-course of pmAAT mRNA induction

In a previous report [5] we showed that the stimulation of mAAT activity by testosterone occurred after a 2–3 h lag period. Consequently, we determined the time-course of pmAAT mRNA induction to ascertain its relationship to the time-course of pmAAT and mAAT induction. Time-course experiments were performed using primary cultures of mini-pig prostate. Dishes of cells were treated with testosterone for the time periods indicated. Cells were

collected, homogenized and mitochondria, cytosol and poly (A^+)RNA isolated. As shown in Fig. 3 pmAAT mRNA increased 30 min after testosterone treatment, and was maximal between 60 and 90 min at a level 3-fold higher than control. By 2 h the pmAAT mRNA concentration had decreased, but then increased again by 4 h. The specific activity of mAAT increased with a much slower time-course. By 6 h mAAT activity was still increasing. The maximum increase in mAAT activity was generally about 3-fold which generally occurred after 24 h. The specific activity of pmAAT activity reached a maximum by 90 min and decreased as mAAT activity increased.

In order to determine if the increase in pmAAT mRNA resulted from a specific effect of testosterone on pmAAT mRNA or was the result of a generalized increase in mRNA, the level of poly(A^+)RNA was measured by hybridization to an oligo(dT) probe. The maximum increase in poly(A^+)RNA was 2-fold and occurred 1 h after testosterone addition. We normalized the change in pmAAT mRNA based on the change in poly(A^+)RNA by calculating the pmAAT mRNA/Poly(A^+)RNA ratio (Fig. 4). The increase in pmAAT mRNA was 3-fold greater than the increase in poly(A^+)RNA which indicated that the response was specific.

Since castration decreased pmAAT mRNA in ventral prostate *in situ* the time-course of the pmAAT mRNA change was followed in mini-pig prostate cell cultures when testosterone was removed from the medium. This deinduction period would provide an estimate of the pmAAT mRNA half life. In these experiments mini-pig cells which had been incubated in medium supplemented with testosterone and 10% FBS were changed to SFRE 199-2 serum free medium without testosterone and incubated for the times indicated (Fig. 5). The level of pmAAT mRNA and pmAAT activity decreased rapidly when testosterone was removed from the medium. We estimated the half-life of pmAAT mRNA to be 1.5 h based on linear regression analysis of the semilog plot of the decay (Fig. 5, inset).

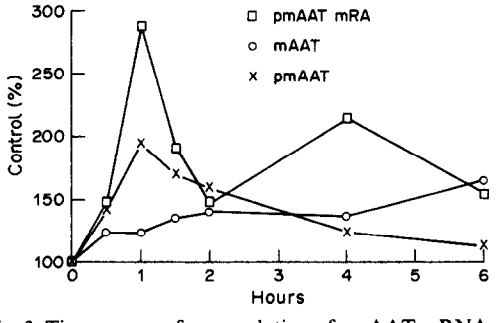

Fig. 3. Time-course of accumulation of pmAAT mRNA and pmAAT and mAAT enzyme specific activity in mini-pig prostate cells with 10^{-8} M testosterone for the indicated times. Quadruplicate sets of dishes of cells were exposed to testosterone for various times. Enzyme specific activities were determined on mitochondrial and cytosol extracts and pmAAT mRNA was determined in poly(A^+)RNA isolated from the free polysome fraction. The data are expressed as fold increase relative to the 0 h time point.

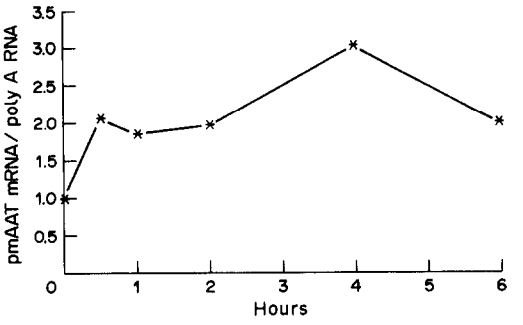

Fig. 4. Ratio of change in pmAAT mRNA and total poly(A^+)RNA in mini-pig prostate cells after treatment with 10^{-8} M testosterone. Triplicate dishes of cells were incubated in testosterone for various periods of time. The cells were collected and the free polysome fraction prepared. Total poly(A^+)RNA was determined by hybridization with a poly (dT) probe and pmAAT mRNA was determined by hybridization with a specific pmAAT mRNA probe. Equal volumes of cell extract were blotted on nitrocellulose filters and hybridized with the appropriate probe. The data are expressed as the ratio of fold increase in pmAAT mRNA to fold increase in poly(A^+)RNA.

DISCUSSION

In an earlier report [6] we proposed a mechanism for testosterone regulation of prostatic citrate production. We proposed that aspartate transamination via mAAT activity is the major source of oxalacetate for citrate synthesis in prostate. Furthermore, we

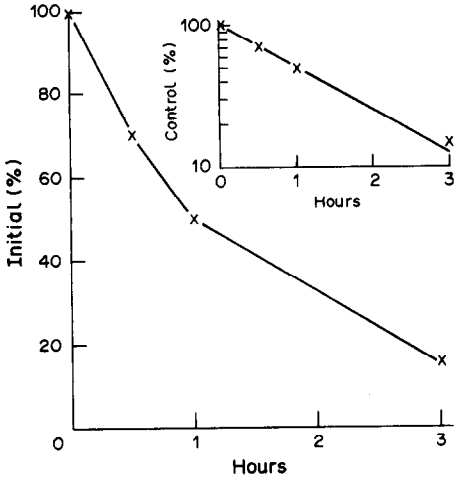

Fig. 5. Estimate of pmAAT mRNA half life in mini-pig prostate cells after deinduction by removal of testosterone from the medium. Mini-pig prostate cells were incubated overnight in medium containing 10^{-8} M testosterone. The next day cells were collected from dishes in groups of 3 at various times. pmAAT mRNA was determined in free polysome extracts by hybridization to a specific pmAAT mRNA probe. The data are expressed as the % of initial (0 h) pmAAT mRNA remaining. The inset is the semilog plot of the same data.

proposed that testosterone regulates citrate production by stimulating prostate mAAT activity. Like many mitochondrial matrix proteins mAAT is derived from a cytosolic precursor (pmAAT) which is imported into mitochondria. It is this pmAAT precursor which is the product of mRNA translation. We proposed that an important action of testosterone in prostate is stimulation of pmAAT biosynthesis which results in an increased level of mAAT and mAAT activity. Our earlier report [6] demonstrated that testosterone treatment resulted in a significant increase in the synthesis of pmAAT in ventral prostate. In this study we investigated the response of pmAAT mRNA steady-state levels to testosterone manipulation.

In order to measure pmAAT levels we used a synthetic 24 base DNA probe. Initially we used a mixed probe with a sequence based on all possible base combinations encoding for rat mAAT at amino acids 272–280. Later another probe was synthesized from the sequence of pmAAT cDNA reported by Mattingly *et al.*[10]. We tested the specificity of the probes using membrane bound and free polysome fractions of rat liver as well as mRNAs and DNA from several other sources. Both probes hybridized to only those preparations which contained pmAAT mRNA. Therefore, we concluded that these synthetic oligonucleotides were specific for pmAAT mRNA.

In addition to *in vivo* studies in rats we also used primary cultures of mini-pig prostates. We have previously reported the advantages of mini-pig prostates over rat ventral prostate for primary cultures [14]. We also reported that cell cultures of mini-pig prostate responded to testosterone with citrate accumulation and stimulation of mAAT activity in a manner similar to rat ventral prostate. Therefore, the effects of testosterone on pmAAT mRNA seen in this study with pig cultures can be extended to rat ventral prostate. More importantly, we believe that these testosterone effects may also be extrapolated to human prostate.

The results of this study showed that testosterone increased the steady-state level of pmAAT mRNA. Moreover, the testosterone effect was specific for pmAAT since the increase in total mRNA as represented by poly(A^+)RNA was 2-fold less than for pmAAT mRNA. The results also demonstrated that the time-course of pmAAT mRNA accumulation was consistent with the increase in pmAAT and mAAT activities. Previously we reported that the time-course for the increase in mAAT activity showed a 2–3 h lag period before the increase began [5]. Results reported here showed that the increase in pmAAT mRNA was maximum by 1 h and preceded the mAAT activity increase by 1 h. This is consistent with the 2–3 h lag in mAAT response. This response is similar to the effect of cAMP and dexamethasone on tyrosine aminotransferase (TAT) mRNA of liver cells [15]. However, one important difference between the pmAAT mRNA time-course reported here and TAT mRNA time-course is that with TAT the mRNA returned to basal level by 3–4 h while in our study pmAAT mRNA remained elevated for 4–6 h in the presence of testosterone.

An increase in the steady-state level of mRNA can be accounted for by transcriptional and post-transcriptional regulation. Page and Parker[16] reported that testosterone increased (60-fold) the steady-state level of the mRNA which codes for the C3 peptide of prostatic binding protein by increasing transcription and by increasing the stability of the C3 mRNA. The increase in stability of C3 mRNA may be the principal effect. As pointed out by Rosen *et al.*[17] modulation of acutely regulated genes, such as enzymes, probably involves a stimulation of transcription through a strong promotor with little or no effect on stability. In contrast, secretory proteins such as prostatic binding protein require chronic regulation to maintain a sustained high level of synthesis and secretion. This chronic regulation is probably attained through the synthesis of extremely stable mRNAs. The results reported here do not allow us to draw conclusions about the effect of testosterone on the stability of pmAAT mRNA. However, the results of deinduction experiments and the estimated half-life of 1.5–2.0 h suggest that pmAAT mRNA is not a particularly stable species. The half-life estimated for pmAAT mRNA is nearly the same as the half-life reported for TAT mRNA [18]. Testosterone induction of pmAAT mRNA shares analogies with dexamethasone and cAMP induction of TAT mRNA in liver and hepatoma cells. Glucocorticoid induction of TAT mRNA involves stimulation of TAT transcription with no effect on the stability of TAT mRNA [15, 18]. We expect that pmAAT mRNA induction is comparable to TAT mRNA induction. The 2–4-fold increase in the steady-state level of pmAAT mRNA is most likely the result of a 2–4-fold increase in pmAAT transcription. Studies are now in progress to determine the effect of testosterone on pmAAT gene transcription and to determine the effect of testosterone on pmAAT mRNA half-life.

We previously reported the time-course of the decrease in mAAT activity in ventral prostate after castration [7]. Those studies showed that 50% of the decrease in mAAT activity occurred during the first 24 h after castration. We suggested, at that time, that those results indicated that mAAT activity was a specific response to castration which preceded the more generalized reduction in protein synthesis. Consequently, we have routinely used castration periods of 24–48 h to eliminate the complicating effects of more generalized changes. The estimated half-life of pmAAT mRNA reported here is consistent with the rapid decrease in mAAT activity we observed after castration. Since the plasma testosterone level is significantly reduced after 24 h of castration these data suggest that the continuous presence of

androgen is required to maintain the pmAAT mRNA level and mAAT activity. The ability of steroid hormones to increase the rate of transcription of hormone induced genes is well documented. For the androgens however, such information has been extrapolated from other steroid regulated systems. This lack of progress has been due in large part to the lack of a model system regulated at the transcriptional level by testosterone in a physiologically meaningful way. Recently, Denison *et al.*[19] reported androgen stimulation of the glucocorticoid response element of the TAT gene. This report suggested that androgens may alter the rate of transcription of specific genes in target tissues by interacting with specific enhancers on genomic DNA. The report by Denison, however, does not necessarily demonstrate the physiological mechanism of action of androgens, since TAT is not induced by testosterone under physiological conditions. Our studies with mAAT represent a specifically identified enzyme which is regulated by an action of androgens on an androgen responsive gene. Moreover, this androgen response has been linked to the functional expression of the target prostate epithelia cell, namely citrate production. These results suggest that the specific response of pmAAT mRNA to testosterone may provide a model for the study of androgen effects on transcription of a specific target gene.

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