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AUTOREGULATION OF ANDROGEN RECEPTOR EXPRESSION IN RODENT PROSTATE: IMMUNOHISTOCHEMICAL AND IN SITU HYBRIDIZATION ANALYSIS

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Summary: Autoregulation of androgen receptor mRNA and protein was investigated by immunohistochemical and *in situ* hybridization techniques. In both mouse and rat prostate, the epithelial cell nuclei were stained with the monoclonal or polyclonal antibodies raised against humann androgen receptor. It was observed that 3 days after castration, nuclear staining of the epithelium was greatly reduced, while androgen treatment restored the staining intensity to a normal level. *In situ* hybridization using an androgen receptor cDNA fragment as probe demonstrated that the change in androgen receptor mRNA level correlated with the change in antibody staining intensity. These data suggested an up-regulation of androgen receptor expression by androgen.

Androgens have a great variety of effects on many target tissues [1]. They act by binding to an intracellular receptor protein [2]. These hormone-receptor complexes are thought to interact with specific DNA sequences and thereby regulate the expression of selected genes. Androgen receptors are found in many organs including reproductive organs [3]. Like normal tissue, prostate cancer, one of the most prevalent of all cancers, has also been known to be responsive to androgen stimulation.

Since the magnitude of the effects of hormones is mainly determined by both the concentration of the hormone and by the intracellular receptor number, it is important to understand the basis of the regulation of androgen receptor expression. It has been reported that the expression of most steroid receptors such as glucocorticoid and estrogen receptors, is down-regulated by

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Abbreviations: DHT, 5α-dihydrotestosterone; SDS, sodium dodecyl sulfate; DTT, dithiothreitol.

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the receptor ligand [4-7]. For androgen receptor, however, autoregulation of androgen receptor expression is still controversial. Kaufman *et al* [8] and Gad *et al* [9] have demonstrated the up-regulation of androgen receptors by androgens in human genital skin fibroblasts by ligand binding assay. On the other hand, Quarmby *et al* [10] have shown a down-regulation of androgen receptor mRNA level in androgen target organs including prostate by Northern blot analysis. These discrepancies seem to be dependent on the techniques used. Furthermore, most biochemical assays measured androgen receptor level with homogenized tissues which included epithelial and stromal cells. In those cases, the interpretation could be complicated because the response to androgens may differ in each cell type.

Since cDNAs which encode full-length rat and human androgen receptors have constructed [11, 12], and also monospecific antibodies against androgen receptors have been made [13], immunohistochemical and *in situ* hybridization techniques can be applied to the study of androgen receptor expression. In this report, we examined the effect of castration on the expression of androgen receptor protein and its mRNA in prostatic tissues by means of immunostaining and *in situ* hybridization techniques. These techniques directly demonstrated the change in androgen receptor content in tissue sections at a cellular level.

MATERIALS AND METHODS

Animals and Tissues: Adult male Spraque-Dawley rats were purchased from Taconic (Germantowin, NY) and adult male Crl:CD-1 (ICR) mice were from Charles River Inc. (Wilmington, MA). The animals were castrated by the scrotal route while under diethyl ether anesthesia. When indicated, they were given daily subcutaneous injections containing 5 mg (for rats) or 1 mg (for mice) of 5α-dihydrotestosterone (DHT) in 0.5 ml sesame oil.

For immunohistochemical analysis, fresh ventral prostates were placed in O.C.T. compound (Miles Laboratories Inc. IN) and immediately frozen on solid CO_2. The frozen tissues were stored at -70°C until use.

For *in situ* hybridization analysis, ventral prostate taken from the animals were immediately fixed with 4% paraformaldehyde for 0.5-2 hours (depending on the size of specimens), embedded in O.C.T. compound and frozen on solid CO_2. The frozen tissues were stored at -70°C until use.

Antisera and Immunoprecipitation: The preparation of monoclonal and polyclonal anti-androgen receptor antibodies has been described previously [13]. The monoclonal antibody was made in rat and the polyclonal antibody was made in rabbit. The antibodies used in this study was raised against the purified human androgen receptor (40% of the N-terminal domain and 25% of the DNA binding domain)-bacterial *trp* E fusion protein and their specificity was confirmed by sucrose gradient centrifugation and double immunoprecipitation assay [13].

To confirm that the antibodies recognize equally both ligand-free and -bound receptors, immunoprecipitation experiments were carried out in the presence or absence of androgen. Linearized pGEM-3Z (Promega, Madison, WI) containing the human androgen receptor cDNA was transcribed by T7 RNA polymerase (Promega) and the RNA was translated in a rabbit reticulocyte lysate

(Promega) in the presence of ^{35}S-methionine (16 µM, 1066 Ci/mM, Amersham). Aliquots of the lysate mixture (50 µl) were then incubated with 0, 10 and 100 nM of DHT for 4 hours at 4°C. Four µl of each translation reaction was diluted with 0.1% nonidet P-40 in phosphate-buffered saline (pH 7.4) and incubated for 1 hour at 4°C with 20 µl of monoclonal antibody N-15 (0.5 mg/ml) or 2 µl of polyclonal antibody (30 mg/ml) (total volume 200 µl), followed by the incubation with 25 µl of anti-rat IgG agarose (Sigma) for monoclonal or 20 µl of immunoprecipitin (Protein A, Amersham) for polyclonal antibody for 1 hour. After centrifugation, the pellet was washed extensively with lysis buffer (150 mM NaCl, 1% nonidet P-40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH 7.5)) and were incubated at 85°C for 10 min in sample buffer (2% SDS, 10% glycerol, 100 mM dithiothreitol (DTT), 5% 2-mercaptethanol, 60 mM Tris-HCl (pH 6.8) and 0.001% bromphenol blue). The eluted proteins were separated by 8% SDS-polyacrylamide gel electrophoresis. The gel was treated with 20% 2,5-diphenyllozaxazole (PPO, New England Nuclear, Boston, MA) in acetic acid, dried, and autoradiographed.

Immunohistochemical Staining of Androgen Receptor: The details of immunostaining with our anti-androgen receptor antibodies have been described previously by Takeda *et al* [3]. Since the monoclonal antibody was made in rat and polyclonal antibody was made in rabbit, the mouse prostate was stained with both monoclonal and polyclonal antibodies while the rat prostate was stained with only polyclonal antibody. Frozen sections, 7 µm thick, from unfixed tissues were fixed in a 4% paraformaldehyde solution and were incubated with the first antibody (15 µg/ml) at 4°C overnight. They were then incubated with 2nd antibody (biotinylated anti-rat IgG for monoclonal and biotinylated anti-rabbit IgG; Zymed Laboratories Inc., San Francisco, CA) at room temperature. This was followed by the incubation with peroxidase-streptavidin (Zymed) at room temperature. Finally, the sections were incubated with substrate solution (0.01% H_2O_2, 0.05% 3,3-diaminobenzidine tetrahydrochloride; Sigma). For control experiments, the immune serum which was absorbed with antigen (the purified androgen receptor fusion protein) was used as first antibody.

***In situ* Hybridization**: The probe used for *in situ* hybridization was a 0.6 kb NruI-Hind III fragment of rat androgen receptor cDNA which was subcloned into pBluscript II SK(+) (Stratagene) at the site of SmaI and Hind III. The specific hybridization of the probe to mouse androgen receptor mRNA has been confirmed by Northern blot analysis [14].

The *in situ* hybridization conditions used in the present study essentially follow those of Simerly *et al* [15] and described precisely in our previous paper [14].

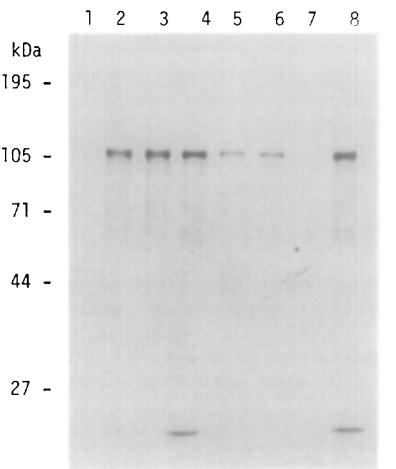
For quantification of autoradiographic signals, 30 alveoli were randomly selected from each group (3-5 animals for each group) and the number of silver grains which appeared in epithelial cells was counted and divided by the total cell number (each alveola contained 200-400 epithelial cells in 10 µm thick section). The background was evaluated by counting within a 50 µm square with no tissue. All values were compared by using Student's t-test for unpaired data.

RESULTS

In the present study, we focused on the epithelial cells, a major component of adult prostate tissue, where most of the AR immunostaining lie on because stromal cells showed heterogeneity in the intensity of staining and *in situ* hybridization signal.

Immunoprecipitation of Translated Androgen Receptor: The specificity of the antibodies used in these experiments was confirmed previously by gradient

centrifugation and binding assay of immunoprecipitated proteins. For further confirmation of whether the antibody can react with receptor protein equally in the presence and absence of androgen, androgen receptor protein derived from *in vitro* transcription and translation of pGEM-3Z vector containing full length human androgen receptor cDNA was incubated with DHT, and immunoprecipitated by the monoclonal and polyclonal anti-androgen receptor antibodies. The immunoprecipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis. The translation from full length human androgen receptor cDNA, which has been shown previously to possess androgen binding activity [11], is composed mainly of a 98-kDa protein. Although the monoclonal antibody was less effective, both monoclonal and polyclonal antibodies precipi-



1. Lysate + DHT - antibody
2. Lysate + DHT + polyclonal antibody
3. Lysate - DHT + polyclonal antibody
4. Lysate only
5. Lysate + DHT + monoclonal antibody
6. Lysate - DHT + monoclonal antibody
7. Lysate - DHT - antibody
8. Lysate only

Fig. 1. Immunoprecipitation of androgen receptor proteins with monoclonal and polyclonal anti-androgen receptor antibodies.

RNA transcribed from pGEM-3Z plasmid containing full length human androgen receptor cDNA was translated in a rabbit reticulocyte lysate in the presence of ^{35}S-methionine. The lysate mixture was incubated at 4°C in the absence or presence of 100 nM DHT. Aliquot of the lysate mixture was directly analyzed by 8% SDS-polyacrylamide gel (lane 4, the lysate after incubation in the presence of DHT; lane 8, the lysate after incubation in the absence of DHT). The receptor cDNA mainly produced a 98 kDa proteins. The remaining mixtures were then immunoprecipitated with the polyclonal (lane 2, in the presence of DHT; lane 3, in the absence of DHT) or with the monoclonal antibody (lane 5, in the presense of DHT; lane 6, in the absence of DHT). For negative controls, the lysate mixture was immunoprecipitated with preimmune rabbit (lane 1) or rat (lane 7) serum. Both polyclonal and monoclonal antibodies reacted the 98 kDa protein and there is no significance difference between immunoprecipitation in the presence and absence of DHT. Also, no difference was observed between 10 nM and 100 nM DHT treatment (Data not shown).

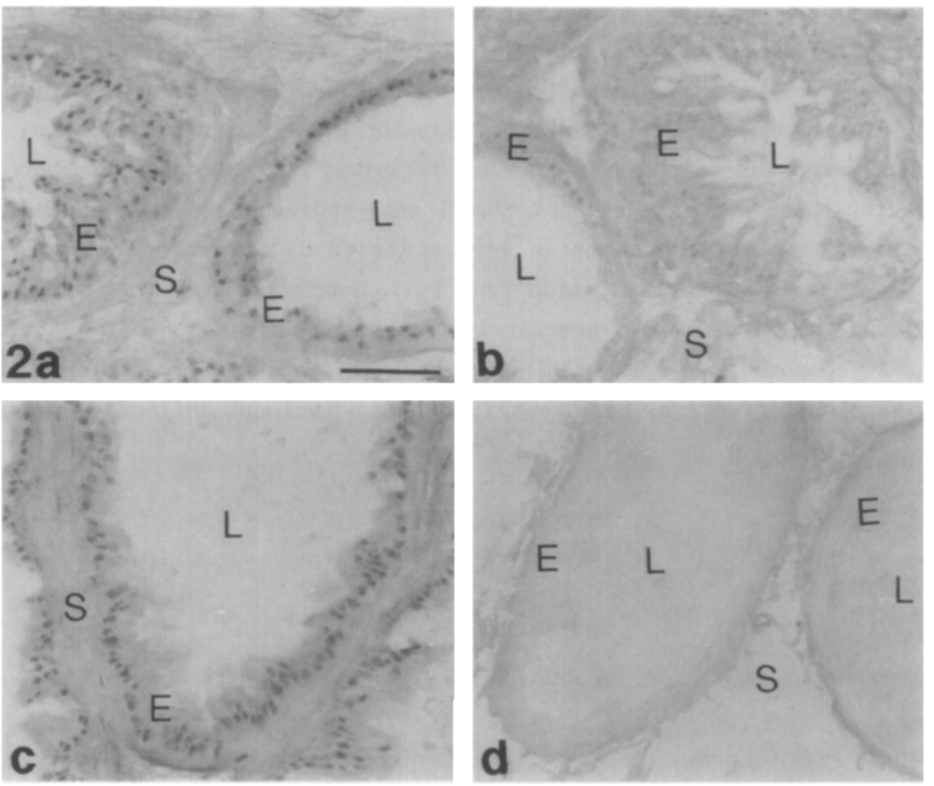


Fig. 2. Immunostaining of mouse prostates after withdrawal and replacement of androgens. Mouse ventral prostate were stained with monoclonal anti-androgen receptor antibody after no treatment (a), and 3 days after castration (b). At 3 days after castration, additional mice were injected daily with DHT 3 days before sacrifice and their prostates were stained with the antibody (c). The control sections were stained with antigen-absorbed antibody (d). Castration greatly reduces staining intensity of epithelial nuclei and the staining is restored by the administration of androgens. No nuclear staining is observed in a control section.
L, lumen; E, epithelium; S, stroma
Bar = 100 μm

tated the major 98-kDa protein and no significant difference in immunoprecipitation was observed in the presence and absence of DHT (Fig. 1).

Effects of Castration on the Immunostaining of Prostates: To examine the effect of androgens on androgen receptor protein synthesis at a cellular level, the ventral prostates from rats and mice castrated or castrated and injected with DHT were stained with anti-androgen receptor antibodies. In normal prostate, positive reactions were found mainly in the nuclei of epithelial cells. Immunohistochemical staining revealed that withdrawal of androgens dramatically reduced the staining intensity in the nuclei. The decrease in the staining intensity was detected 1 day after castration and reached the lowest level 2 to 3 days after castration. Androgen administration in castrated animals restored the nuclear staining to a normal level after 2 to 3 days (Fig. 2). Both monoclonal and polyclonal antibodies

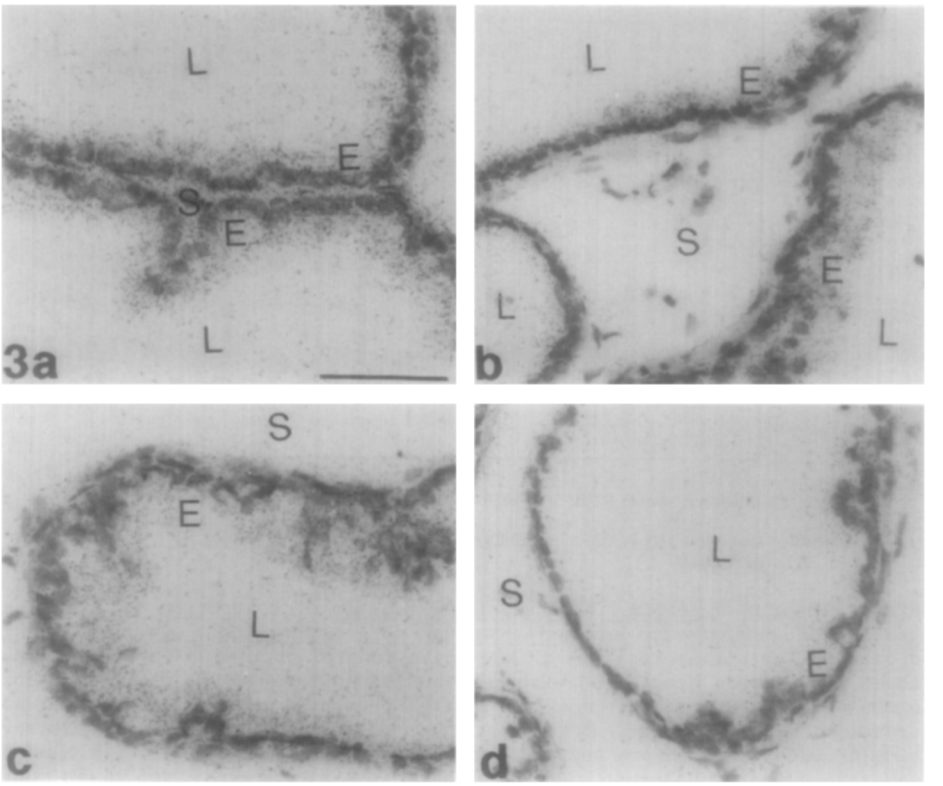


Fig. 3. *In situ* hybridization analysis of mouse prostate after withdrawal and replacement of androgens. Mouse ventral prostate sections were hybridized with ^{35}S-labeled anti-sense RNA probe after no treatment (a), and 3 days after castration (b). At 3 days after castration, additional mice were injected daily with DHT 3 days before sacrifice and their prostate sections were hybridized with anti-sense probe (c). The control sections were hybridized with ^{35}S-labeled sense RNA probe (d). Castration reduces the intensity of hybridized signals and it is restored by the administration of androgens. A few silver grains were observed in control sections. For quantification of the autoradiograms, see Fig. 4.
L, lumen; E, epithelium; S, stroma
Bar = 100 μm

produced essentially the same pattern of staining, and no significant difference in the staining pattern was detected between mouse and rat prostates.

***In Situ* Hybridization Analysis of Androgen Receptor mRNA Expression**: To investigate the change of androgen receptor mRNA at a cellular level, ventral prostate sections from normal, castrated and DHT-injected animals were subjected to *in situ* hybridization. The sections were hybridized with ^{35}S-labeled anti-sense RNA probes and the number of silver grains which appeared in the epithelial cells were counted. Although the number of silver grains varied much among the alveoli, the result is largely in agreement with the immunostaining data; castration reduced the receptor mRNA level by 25% and

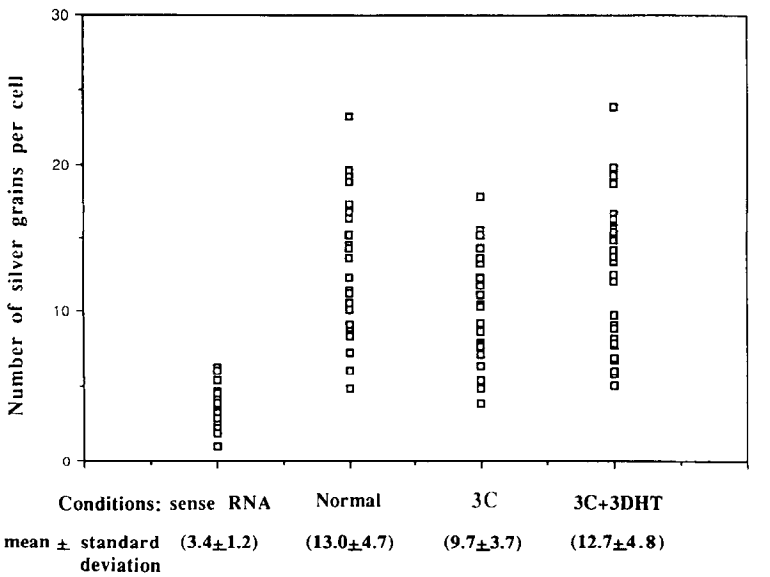


Fig. 4. *In situ* hybridization analysis of effects of castration and administration of androgen on androgen receptor mRNA expression in mouse ventral prostate. The tissue sections of the mouse ventral prostates from following three groups were hybridized with ^{35}S-labeled anti-sense RNA probe and were processed for autoradiography; the ventral prostates of adult mice after no treatment (Normal), 3 days after castration without (3C) or with administration of DHT 3 days before sacrificing (3C+3DHT). Open rectangle (□) represents an average number of silver grains per cell from one alveola. In control experiments, the sections were hybridized with ^{35}S-labeled sense RNA. Thirty alveoli, which contain about 200 -400 epithelial cells in a section of 10 μm thickness, were randomly selected from each group and the average number of silver grains per cell was plotted. Emulsion back ground was evaluated by counting grains within a 50 μm square with no tissues. As the background level was quite low (about 0.5 grains per cell)(the typical size of epithelial cell is about 5 μm by 10 μm square), grain density in the epithelial cells was reported in absolute value without subtraction of any assumed background. The average of each group was described under the graph (mean ± standard deviation). Although the number of silver grains varied much among the alveoli, the results indicate the 'up-regulation' of the receptor mRNA by androgens ($P<0.05$ for normal compared to 3C and for 3C compared to 3C+3DHT). For Autoradiograms, see Fig. 3.

the administration of androgen after castration increased it to a normal level (Figs. 3, 4).

DISCUSSION

The specificity of our antibodies has been confirmed previously by biochemical methods [13]. It was also shown in the present study that the antibody reacted the receptor equally in the presence or absence of androgen.

Our immunostaining and *in situ* hybridization data indicated that in rat and mouse prostate, androgen-withdrawal decreased both androgen receptor content and androgen receptor mRNA level, and that the injection of androgen restored normal levels, a process termed 'up-regulation'. These results

coincide well with previous studies. Gad *et al* [9] have demonstrated by ligand-binding assay that 24 hours exposure of genital skin fibroblasts to DHT or R1881 (synthetic androgen) increased androgen receptor by two fold and the increase was inhibited by actinomyucin D or cyloheximide, suggesting that transcription and translation were necessary for the up-regulation of androgen receptor expression. In addition to the *in vitro* experiments, up-regulation of androgen receptor expression has been demonstrated in rat prostate by ligand-binding assay [16] and by steroid autoradiographic analysis [17]. In the present study, the changes in immunostaining intensity after castration or injection of androgen were much greater than the changes in mRNA level detected by *in situ* hybridization. This suggests that expression of androgen receptor may be regulated by androgens at both the transcriptional and post-transcriptional levels. In fact, transcriptional and posttranscriptional regulation have been demonstrated in other steroid hormone receptors such as glucocorticoid receptor [18] and estrogen receptor [7].

However, Northern blot data of Quarmby *et al* [10] in rat prostate have shown a different result, down-regulation; the amount of androgen receptor mRNA increased by androgen withdrawal and decreased below the control level after androgen stimulation. Our preliminary Northern blot data (unpublished data) also showed the same tendency, down-regulation. There might be two alternative explanation for the discrepancy between Northern blot and immunostaining or *in situ* data. One possibility is that the stromal cells respond to androgens in different way, which could complicate the interpretation of the biochemical analysis. The other possibility is as follows. The Northern blot data are usually normalized with the total amount of mRNA, which is often confirmed by constitutively expressed mRNA species such as actin. Normalized Northern blot data, therefore, can only show the proportion of a specific mRNA in the total RNAs. Moreover, the prostate is a highly androgen-dependent organ. Although cell death is not obvious in the first 3 days after castration [2 16], deprivation of androgen by castration causes a variety of changes in the prostate: a dramatic disintegration of cytoplasmic structures [19] such as the endoplasmic reticulum and mitochondria, a decrease in total RNA content and total protein [20, 21], and a loss of many species of mRNAs [22]. In this case, an interpretation of Northern blot data is complicated; an increase in the intensity of the specific band in the blot analysis does not necessarily indicated an increase in the amount of the mRNA per cell. Thus, it is possible that the apparent down-regulation in the blot analysis indicates the relative persistence of androgen receptor mRNA after castration. The rate of decrease of androgen receptor mRNA may be lower than that of other androgen-responsive mRNAs. Similarly, the rate of increase of androgen receptor mRNA after androgen replacement in castrated animals may be

lower than that for other androgen responsive mRNAs. The *in situ* hybridization technique, on the other hand, allows the direct quantification of a specific mRNA at a cellular level without need of normalization. At the moment, we can not figure out which possibility is the case. The experiments using homogeneous cultured cells are being done in our laboratory.

In conclusion, the present study demonstrates by immunohistochemical and *in situ* hybridization techniques that androgen receptor protein and mRNA are up-regulated by androgens.

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