tively high amounts for further structural characterization as well as elucidation of the molecular basis for their antifungal activity.

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REFERENCES

[1] Carr, J.P. and Klessig, D.F. (1989) In: Genetic Engineering: Principles and Methods (J.K. Setlow, Ed.) pp. 65–109, Plenum Press, New York.
[2] Pierpoint, W.S. and Tatham, A.S. (1987) Physiol. Mol. Plant Pathol. 31, 291–298.
[3] Singh, N.K., Bracker, C.A., Hasegawa, P.M., Handa, A.K., Buckel, S., Hermodson, M.A., Pfankoch, E., Regnier, F.E. and Bressan, R.A. (1987) Plant Physiol. 85, 529–536.
[4] Cornelissen, B.J.C., Hooft van Huijsduijnen, R.A.M. and Bol, J.F. (1986) Nature 321, 531–532.
[5] Pierpoint, W.S., Jackson, P.J. and Evans, R.M. (1990) Physiol. Mol. Plant Pathol. 36, 325–338.
[6] Bryngelsson, T. and Gréen, B. (1989) Physiol. Mol. Plant Pathol. 35, 45–52.
[7] Jacobsen, S., Mikkelsen, J.D. and Hejgaard, J. (1990) Physiol. Plant. 79, 554–562.
[8] Leah, R., Tommerup, H., Svendsen, I. and Mundy, J. (1991) J. Biol. Chem. 266, 1564–1573.
[9] Richardson, M., Valdes-Rodriguez, S. and Blanco-Labra, A. (1987) Nature 327, 432–434.
[10] Roberts, W.K. and Selitrennikoff, C.P. (1990) J. Gen. Microbiol. 136, 1771–1778.
[11] Hejgaard, J. and Bjørn, S.E. (1985) Physiol. Plant. 64, 301–307.
[12] Boisen, S., Andersen, C.Y. and Hejgaard, J. (1981) Physiol. Plant. 52, 167–176.
[13] Soor, S.K. and Hincke, M.T. (1990) Anal. Biochem. 188, 187–191.
[14] Hejgaard, J. and Gibbons, G.C. (1979) Carlsberg Res. Commun. 44, 21–25.
[15] Svendsen, I., Hejgaard, J. and Chavan, J.K. (1984) Carlsberg Res. Commun. 49, 493–502.
[16] Iyengar, R.B., Smits, P., van der Ouderaa, F., van der Wel, H. and van Brouwershaven, J. (1979) Eur. J. Biochem. 96, 193–204.
[17] Yphantis, D.A., Dainko, J.L. and Schlenk, F. (1967) J. Bacteriol. 94, 1509–1515.

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cDNA cloning of a human androgen-induced mRNA exhibiting an early and protein synthesis-independent induction

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The detailed mechanism of action of androgens remains unknown. We have used an androgen-dependent human prostate cancer cell line and a subtractive cDNA hybridisation strategy to enrich for androgen-dependent sequences. This yielded a cDNA clone which exhibits the characteristics of a primary *trans*-activated target for androgens. This androgen-regulated gene encodes a polyadenylated 4.5 kb mRNA which is induced 30–50-fold within 3 h of treatment with 10 nM dihydrotestosterone. The induction does not require continued protein synthesis as it is maintained in the presence of protein synthesis inhibitors.

Androgen-induced; Dihydrotestosterone; mRNA; Gene

1. INTRODUCTION

The molecular mechanisms of androgen action remain poorly understood despite considerable attention. The receptor for androgens has been cloned and sequenced [1] and shown to belong to the steroid/thyroid/retinoid superfamily of transcriptional activators [2], although no primary gene targets for androgen action have yet been identified. Some androgen responsive genes have been identified, but the long time courses of induction suggests that these genes are not primary targets for androgen action but are *cis*-regulated by the products of early genes [3,4]. In at least one system, liganded androgen receptor is capable of acting as a transcriptional activator through a permissive nucleotide response element in the long terminal section of the mouse mammary tumour virus genome. This response element is also activated by liganded glucocorticoid and progesterone receptors [5,6], thus throwing doubt on the existence of a unique response element for the liganded androgen receptor.

The LNCaP human prostate cancer cell line is dependent upon androgens for continued growth [7]. Withdrawal of all androgens from these cells causes a complete cessation of cell growth within 3 days. We have used these cells in a subtractice cDNA hybridisation system [8] to enrich for sequences that are rapidly induced by dihydrotestosterone (DHT) in an attempt to isolate genes which fulfil the requirements for *trans*-activated androgen targets. Using this technique we have isolated the cDNA for a gene which has all the properties of a primary response gene for androgens; its mRNA is induced within 3 h of treatment with physiological concentrations of DHT and its induction is not abolished by co-treatment with cycloheximide or puromycin. The induction is abolished by treatment with actinomycin D. The specificity of the induction of this mRNA is demonstrated by the lack of effect of progesterone or dexamethasone, indicating a unique response element for androgens in the 5' flanking region of this gene.

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2. MATERIALS AND METHODS

Androgen-dependent LNCaP cells were routinely maintained in RPMI 1640 medium (Flow Laboratories, UK) supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine and 10 nM DHT at 37°C in 95% air/5% CO_2. Cells were free from mycoplasma contamination. Total cellular RNA was extracted by the guanidium isothiocyanate method, with the selection of mRNA by poly-A^+ chromatography on oligo-dT cellulose columns.

mRNA was isolated by 2 rounds of oligo-dT chromatography from LNCaP cells that had been treated for 12 h with 10 nM DHT in ethanol vehicle such that the ethanol concentration remained at 0.1% v/v. Control cells received ethanol vehicle alone. Prior to this 12 h treatment the cells were withdrawn from all androgenic growth stimuli by culturing the cells for 7 days in RPMI 1640 supplemented with 10% dextran-coated charcoal-stripped FCS (DCC-FCS) [9]. 10 μg of the mRNA from LNCaP cells treated for 12 h with 10 nM DHT (+A cells) was then used to synthesise single stranded cDNA, weakly labelled by the incorporation of [^{32}P]dCTP (Amersham) [10]. Following purification by spun-column chromatography on Sephadex G-50 (Pharmacia), this cDNA was hybridised to a 10-fold mass excess of mRNA from control LNCaP cells (−A cells) at 68°C for 22 h [11]. Following the hybridisation, single stranded cDNA was removed from cDNA:mRNA heteroduplexes by hydroxylapatite chromatography [12], and after mRNA alkaline hydrolysis and purification, the cDNA was re-hybridised to another 10-fold mass excess of mRNA from −A

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cells, again at 68°C for 22 h. Following another round of hydroxylapatite chromatography the subtracted cDNA was purified by Sephadex G-50 spin columns, labelled to high specific activity with [^{32}P]dCTP by the Klenow fragment/random primer method [13] and used to probe 100 000 plaques of a +A directional cDNA library constructed in Lambda ZAP (Stratagene). Duplicate nylon filter lifts (Hybond-N, Amersham) were hybridised overnight at 42°C in 0.05 M Tris, pH 7.5, containing 50%, v/v formamide, 0.2% w/v polyvinylpyrrolidone, 0.2% w/v Ficoll, 1 M NaCl, 0.1% w/v sodium pyrophosphate, 10% w/v dextran sulphate, 1% w/v SDS, and 50 μg/ml denatured salmon sperm DNA. The filters were then washed in 0.2 × SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.5) with 0.1% w/v SDS at 55°C for 1 h and exposed to X-ray film overnight at −70°C with intensifying screens. A single, strongly hybridising plaque was identified, picked and rescreened using the same subtracted probe. Positives from the secondary screening were plaque-purified and in vivo subcloned into the pBluescript plasmid [14]. From this plasmid was isolated a 1.1 kb cDNA insert which was purified by low-melting-point agarose electrophoresis and used in the subsequent Northern analyses.

For Northern blots, 10 μg aliquots of mRNA, derived from LNCaP cells under different experimental conditions and purified by 2 rounds of poly-A$^+$ chromatography, were fractionated through 1% denaturing formaldehyde agarose gels and transferred to Hybond-N. Hybridisation conditions for the Northern blots were as above for the plaque screening, using random primer-labelled insert cDNA. Filters were washed at 55°C in 0.2 × SSC with 0.1% w/v SDS for 1 h and exposed to X-ray film at −70°C with intensifying screens for 48 h. Following exposure, the blots were stripped and rehybridised to a β-actin cDNA.

3. RESULTS AND DISCUSSION

The suitability of the LNCaP cell line as an in vitro model for androgen dependence is illustrated in Fig. 1. After the withdrawal of all androgens by culturing the cells in medium containing DCC-FCS the cells stop dividing and remain in stationary phase over the 9 days

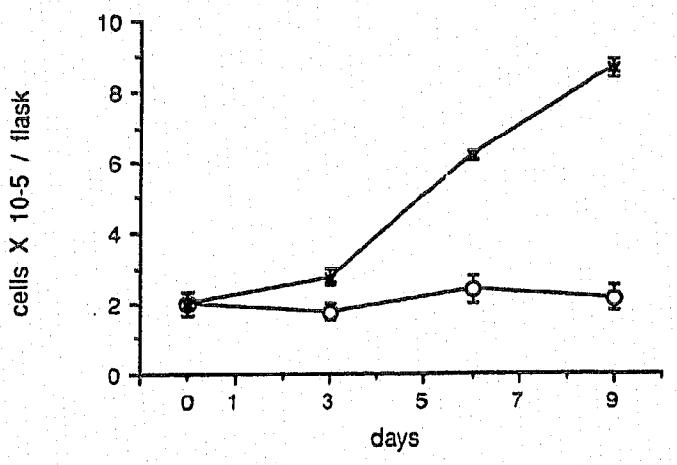

Fig. 1. Time course of the response of LNCaP human prostate cancer cells to 10 nM DHT. Cells were seeded (10^5 cells per 25 cm^2 flask) in routine maintenance medium and allowed to attach for 24 h. This medium was then removed and replaced with medium containing 10% DCC-FCS and the cells cultured for a further 7 days. The medium was changed 3 times during this period. After the 7 day withdrawal period, either 10 nM DHT (X--X) or ethanol vehicle (○--○) were added to the cells and the experiment continued for 9 days with medium and drug changes every 48 h. Quadruplicate flasks of cells were trypsinised and viability counted in a haemocytometer after staining with Trypan blue. The data presented are the means ±SD.

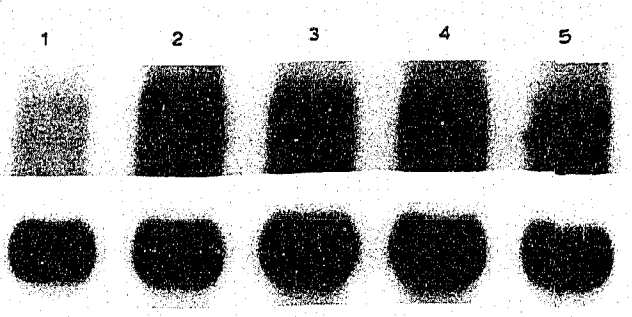

Fig. 2. Northern analysis of 10 μg aliquots of mRNA showing the effect of protein or RNA synthesis inhibitors on the expression of an androgen-induced gene. Lane 1, control cells withdrawn from androgens for a 7 day period. Lane 2, cells withdrawn from androgens for 7 days and then treated with 10 nM DHT for 12 h. Lane 3, cells withdrawn from androgens for 7 days and then treated with 10 nM DHT and 1 μg/ml cycloheximide for 12 h. Lane 4, cells withdrawn from androgens for 7 days and then treated with 10 nM DHT and 50 μg/ml puromycin for 12 h. Lane 5, cells withdrawn from androgens for 7 days and then treated with 10 nM DHT and 2 μg/ml actinomycin D for 12 h.

of the growth experiments. However, the cells that are treated with 10 nM DHT recover, after a lag phase of 2–3 days, and grow thereafter with a doubling time of approximately 4 days. These cells wil continue to divide at this rate for as long as 10 nM DHT is provided.

The Northern blot illustrated in Fig. 2 clearly shows the induction of the early androgen-dependent gene over a 12 h period in the LNCaP cells treated with 10 nM DHT alone or 10 nM DHT with the protein synthesis inhibitors cycloheximide and puromycin. This demonstrates that protein synthesis is not required for the DHT-associated increase in this transcript, implying that this induction is a primary response to the addition of physiological concentrations of DHT and does not require prior induction of gene expression at either the transcriptional or translational level. The induced mRNA is not a single band but a doublet with the upper, major band running at approximately 4.5 kb and the fainter, lower band running at approximately 4.4 kb. Scanning densitometry revealed that the fold induction of this transcript is between 30- and 50-fold over the untreated control cells. The induction is abolished by the addition of the RNA synthesis inhibitor actinomycin D.

The time course of the induction, illustrated in Fig. 3, demonstrates that this is indeed an early transcriptional response of these cells to androgens. The transcript appears to be fully induced within 6 h of treatment with 10 nM DHT and does not increase in magnitude with longer exposure to the hormone, indicating full occupancy of the 5' hormone response element by the liganded androgen receptor within that time.

The usefulness of this clone for studying the molecular mechanisms of androgen action is confirmed by the data in Fig. 4. This Northern blot shows that the induction of the 4.5 kb transcript is unique to DHT and is not seen upon treatment of the cells with either 10 nM pro-

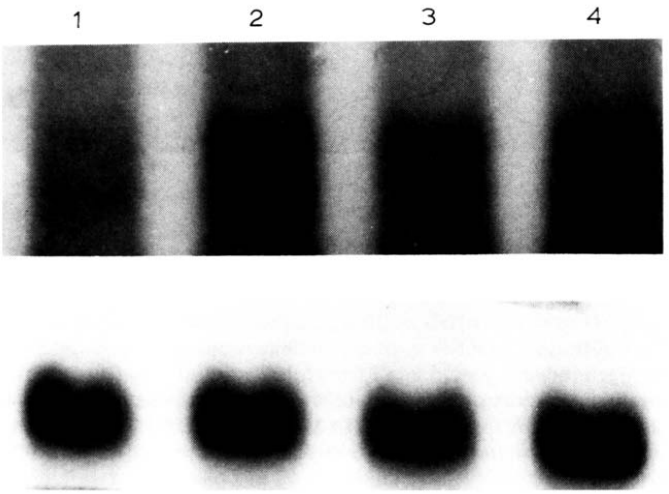

Fig. 3. Northern analyis of 10 μg aliquots of mRNA showing the time course of induction of the androgen-induced gene. Lane 1, control androgen withdrawn cells. Lane 2, androgen withdrawn cells treated with 10 nM DHT for 3 h. Lane 3, androgen withdrawn cells treated with 10 nM DHT for 6 h. Lane 4, androgen withdrawn cells treated with 10 nM DHT for 12 h.

gesterone or 10 nM dexamethasone. These data suggest that this induction is not due to a mouse mammary tumour virus-related hormone response element but to a discrete androgen response element.

The current model of action of members of the steroid/thyroid/retinoid superfamily proposes the very early regulation of one or a small number of transcriptionally activated genes. The expression of these in turn regulates the expression of later genes in a cell and/or tissue specific manner, leading to cell growth or differentiation [15]. Supporting evidence for this model has recently been provided by the cloning of an early retinoic acid-regulated gene in F9 murine teratocarcinoma cells [16]. This gene encodes a homeobox containing protein thought to be centrally important in the retinoic acid-induced differentiation of these cells [17].

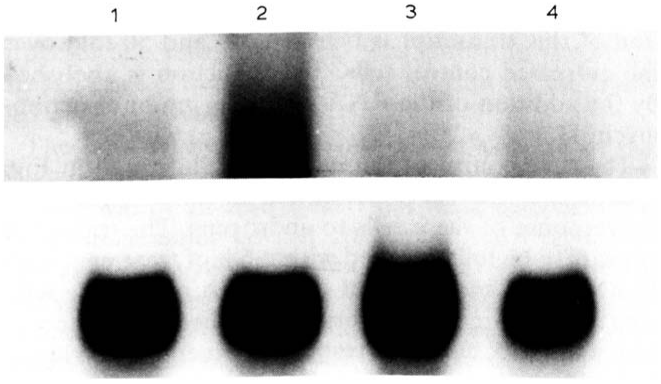

Fig. 4. Northern analysis of 10 μg aliquots of mRNA showing the unique responsiveness of the LNCaP cell line to DHT. Lane 1, control androgen withdrawn cells. Lane 2, androgen withdrawn cells treated with 10 nM DHT for 12 h. Lane 3, androgen withdrawn cells treated with 10 nM progesterone for 12 h. Lane 4, androgen withdrawn cells treated with 10 nM dexamethasone for 12 h.

The function of this early androgen-induced gene is unknown at present but sequencing and expression studies should allow us to examine the role of androgens in other androgen-sensitive systems such as the isolated human sebaceous gland [18] and hair follicle [19].

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REFERENCES

[1] Chang, C., Kokontis, J. and Liao, S. (1988) Proc. Natl. Acad. Sci. USA 85, 7211–7215.
[2] Evans, R.M. (1988) Science 240, 889–895.
[3] Parker, M.G., Needham, M. and White, R. (1980) J. Biol. Chem. 256, 6996–7001.
[4] Chen, Y.H., Pentecost, B.T. and Teng, C.T. (1987) Mol. Endocrinol. 1, 707–716.
[5] Scheidereit, C., Geisse, S., Westphal, H.M. and Beato, M. (1983) Nature 304, 749–752.
[6] Cato, A.C., Henderson, D. and Ponta, H. (1987) EMBO J. 6, 363–368.
[7] Horoszewicz, A.B., Leong, S.S., Kawinski, E., Karr, J.P., Rosenthal, H., Ming Chu, T., Mirand, E.A. and Murphy, G.P. (1983) Cancer Res. 43, 1809–1818.
[8] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning – a Laboratory Manual, Cold Spring Harbor Laboratory Press.
[9] Leake, R.E., Freshnet, R.I. and Munir, I. (1987) in: Steroid hormones – a practical approach (Green, B. and Leake, R.E. eds) pp. 213–214, Irl Press, Oxford.
[10] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning – a Laboratory Manual, Cold Spring Harbor Laboratory Press.
[11] Sargent, T.D. (1987) in: Methods in Enzymology, Volume 152, Guide to molecular cloning techniques (Berger, S.L. and Kimmel, A.R. eds) pp. 427–430, Academic Press.
[12] Sargent, T.D. (1987) in: Methods in Enzymology, Volume 152, Guide to molecular cloning techniques (Berger, S.L. and Kimmel, A.R. eds) pp. 430–431, Academic Press.
[13] Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.
[14] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning – a Laboratory Manual, Cold Spring Harbor Laboratory Press.
[15] Rories, C., Lau, C.K., Fink, K. and Spelsberg, T.C. (1989) Mol. Endocrinol. 3, 991–1001.
[16] La Rosa, G.L. and Gudas, L.J. (1988) Proc. Natl. Acad. Sci. USA 85, 329–333.
[17] La Rosa, G.L. and Gudas, L.J. (1988) Mol. Cell Biol. 8, 390–391.
[18] Ridden, J., Ferguson, D. and Kealey, T. (1990) J. Cell Sci. 95, 125–136.
[19] Philpott, M.P., Green, M.R. and Kealey, T. (1990) J. Cell Sci. 97, 463–471.