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Cloning of cDNA sequences of hormone-regulated genes from the MCF-7 human breast cancer cell line

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ABSTRACT.

cDNA clones corresponding to a mRNA whose level is rapidly increased by addition of oestradiol to the culture medium have been isolated by differential screening of a cDNA library from the breast cancer cell line MCF-7, which contains oestrogen receptors. Such clones will be useful in studies of the DNA sequences required for hormonal induction and to determine whether expression of the corresponding gene is in any way related to the cancerous state. We have also obtained a cDNA clone for a messenger whose level is apparently decreased by steroid hormones.

INTRODUCTION.

The MCF-7 cell line derived from a human breast cancer contains both oestrogen and progesterone receptors (1, 2), and previous work has shown that the levels of some MCF-7 cellular messengers and proteins are under the control of oestrogens (3-7). This system is thus particularly suited for studies of the molecular basis of steroid hormone control : hormone-responsive genes once cloned can be reintroduced into their parent cell in culture and *in vitro* mutagenesis techniques used to define control regions at the DNA level. The availability of cloned hormone responsive genes from MCF-7 cells could also lead to a better understanding of the role of oestrogen in breast cancer : tumors which contain oestrogen receptors may often be treated successfully by endocrine therapy or anti-oestrogen administration (for reviews see Ref. 8). We describe here, as a first step in our analysis of MCF-7 hormone inducible genes, the isolation of a double-stranded cDNA (ds-cDNA) clone representing an MCF-7 RNA whose level is increased by oestradiol treatment. We have also obtained a ds-cDNA clone for a messenger whose level is apparently decreased by steroid hormones.

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MATERIALS AND METHODS

Cell culture

MCF-7 cells were obtained through H. Rochefort (Montpellier, France), and were routinely maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco) and 0.6 μg/ml insulin (Sigma). Serum was stripped of endogenous steroid hormones by treatment with dextran-coated charcoal as described by Horwitz and McGuire (2). When cells were to be treated with stripped serum, confluent cells were plated at a two-fold dilution on day 1 into medium containing stripped serum, and on days 2-10 the cells were washed twice with phosphate-buffered saline (PBS) and re-fed fresh medium. Nafoxidine (a gift from Upjohn), when used, was added to the stripped medium at 1 μM; in this case, treatment of the cells with stripped medium was for 7 days. Oestradiol, when used, was added to stripped medium at 10 nM. Labelling of cells with [^{35}S]-methionine was carried out using the technique described by Westley and Rochefort (3).

Preparation of RNA samples

Total RNA was prepared by homogenising cells in urea-SDS and precipitation with LiCl following the protocol of Auffray and Rougeon (9). Cytoplasmic RNA was prepared by washing cell monolayers once in PBS, once in 0.15 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA (STE), and scraping the cells into cold STE. Cells were pelleted, resuspended in cold STE and repelleted. They were suspended in cold 10 mM NaCl, 1 mM $MgCl_2$, 10 mM Tris-HCl pH 7.5, and NP40 added to 0.5%. After 2-5 minutes on ice, nuclei were pelleted by centrifugation and the supernatant added to an equal volume of cold STE containing 2% SDS. This solution was phenol-chloroform extracted and precipitated with ethanol. The material was reprecipitated twice with ethanol. RNA gels were run using either methyl mercuryhydroxide (12) or formaldehyde (13) as the denaturing agent, and were transferred either to diazobenzyloxymethyl (DBM)-paper (14) or to nitrocellulose sheets (15). Nitrocellulose filters were dehybridised and rehybridised as described by Thomas (15). Hybridization to nick-translated probes (16) and washing conditions were as in (16).

Preparation and screening of cDNA library

Total MCF-7 RNA was subjected to one cycle of oligo(dT) cellulose chromatography, and the polyA(+) RNA used to make cDNA and double-stranded cDNA (ds-cDNA) as previously described (10). The ds-cDNA was C-tailed and annealed to plasmid pBR322 G-tailed at its PstI site, and the

material used to transform E.coli C600 to tetracycline resistance. Colonies were stored at -20°C in the wells of microtitration plates containing "freezing medium" as described by Gergen et al. (11). Colonies stored in microtitration plates were inoculated onto tetracycline containing agar plates in duplicate arrays of 8 x 12 and grown overnight before transfer by contact to filters of Whatman 540 paper. Filters were prepared for hybridisation by the technique of Gergen et al. (11), with modifications (P.M., manuscript in preparation). Filters were hybridised for 92 h at 40°C in batches of 40 in 160 ml of 50 % formamide, 5 x SSC, 0.5 mg/ml denatured salmon sperm DNA with 8 x 10^7 cpm of [^{32}P]-labelled cDNA prepared from MCF-7 RNA by reverse transcription (10). Three radioactive deoxynucleotide triphosphates were used at about 400 Ci/mmol (Amersham). Washing was in 50 % formamide - 5 x SSC at 40°C, followed by several washes in 2 x SSC at room temperature. Filters were exposed to Fuji RX film for 12 days at -80°C.

RESULTS AND DISCUSSION

Construction and screening by differential hybridisation of an MCF-7 cDNA library.

The strategy we decided to employ for cloning cDNA sequences of MCF-7 hormone-regulated genes was to prepare a cDNA library from RNA of cells grown in medium supplemented with 10% fetal calf serum (normal medium); this serum contains steroid hormones. The library would then be screened, using a filter-bound colony hybridisation technique, with two different probes. The first probe would correspond to RNA from cells grown in normal medium, the second probe to RNA from cells grown in medium containing serum stripped of steroid hormones by dextran-coated charcoal treatment (stripped medium), and containing in addition the antioestrogen nafoxidine. Colonies which showed differential hybridisation to the two probes should contain plasmids harboring double-stranded cDNA (ds-cDNA) inserts representing MCF-7 hormone-regulated RNAs. The MCF-7 cells used in our study were obtained from H. Rochefort, and we wished to check both that they continued to show a response to oestrogens under culture conditions in our lab, and that our protocol for stripping serum of endogenous hormones was working effectively. We thus repeated some work done previously by Westley and Rochefort (3), who showed that MCF-7 cells secrete into the medium a 46,000 dalton protein when cultured in the presence of oestrogens, but that this protein

disappears when cells are maintained in medium stripped of oestrogens. We maintained MCF-7 cells either in normal medium or in stripped medium + nafoxidine for 6 days (see Materials and Methods) before harvesting the bulk of the cells for preparation of RNA for cloning and making probes. A small sample of cells maintained in either normal or stripped medium was labelled for 6 h with [^{35}S]-methionine, before the culture medium was collected and analysed on an SDS-polyacrylamide gel. The results are shown in Fig. 1. Cells grown in normal medium secrete large amounts of the 46,000 dalton protein (lane b), while cells maintained in stripped medium + nafoxidine do not (lane a). We found it useful to test each batch of stripped serum in this fashion; some batches gave an unsatisfactory response in this assay and were discarded.

Following the satisfactory results of this experiment, we subjected the RNA of the cells grown in normal medium to one cycle of oligo(dT) cellulose chromatography, and used some of the polyA(+) RNA obtained to make cDNA and double-stranded cDNA (ds-cDNA) by published methods (10). The ds-cDNA was C-tailed and annealed to plasmid pBR322 G-tailed at its PstI site, and the material used to transform E.coli C 600 to tetracycline resistance. In this way about 4000 tetracycline resistant colonies were obtained. Random sampling suggested that at least 80 % of these

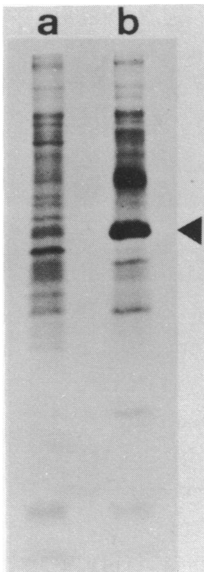

Fig. 1 : Hormonal response of MCF-7 cells. Cells were maintained in either normal or stripped medium plus nafoxidine for 6 days before labelling for 6 hours with [^{35}S]-methionine. The culture medium was removed and analyzed on an SDS polyacrylamide gel. Lanes (a), (b): labelled culture medium from cells maintained in stripped and normal medium, respectively. An arrowhead indicates the migration of the 46000 dalton protein discussed in the text.

colonies harbored plasmids containing ds-cDNA inserts excisable by PstI. The colonies were repicked individually into wells of microtitration plates containing "freezing medium", and stored frozen at -20°C (11). For analysis by differential hybridisation, bacteria from the microtitration plates were inoculated in duplicate ordered arrays onto agar plates containing tetracycline, and allowed to grow overnight at 37°C. Colonies were then transferred to Whatman 540 paper. The duplicate filters were hybridized to [^{32}P]-labelled cDNA transcribed from polyA(+) RNA using reverse transcriptase. One probe was made from RNA isolated from MCF-7 cells maintained in medium containing 10% foetal calf serum and thus oestradiol [(+) probe]. A second probe was made from RNA of cells grown in medium containing serum stripped of steroid hormones by dextran-coated charcoal treatment (2) (stripped medium), and also containing the antiestrogen nafoxidine at 1 μM [(-) probe]. While the majority or filter-bound colonies gave identical signals with (+) or (-) probes, twelve colonies showed preferential hybridisation to one or the other probe. Of these, four colonies hybridised more strongly to the (+) probe than to the (-) probe; an example is shown in Fig. 2, panel B (see empty arrowheads). The remaining eight colonies hybridised more strongly to the (-) probe (see filled in arrowheads Fig. 2, panel A). The diffe-

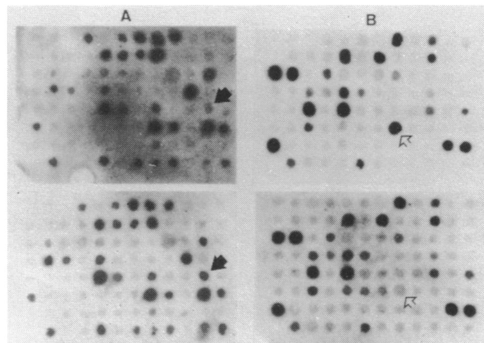

Fig. 2 : Identification by differential hybridisation of bacteria harboring ds-cDNA inserts representing hormonally-regulated MCF-7 RNAs.
Panel A : a pair of duplicate filters hybridised to cDNA transcribed from RNA of MCF-7 cells maintained in normal medium (see text) [top filter, (+) probe] or stripped medium + 1 μM nafoxidine [bottom filter, (-) probe]. Filled in arrowheads indicate a colony which responds more strongly to cDNA from nafoxidine-treated cells. Panel B : a second pair of duplicate filters hybridised as above. Empty arrowheads indicate a colony which responds more strongly to cDNA from cells maintained in the presence of oestradiol (in normal medium).

rence in response to the (+) and (-) probes was much greater for the four colonies (plasmids pS1-4) that hybridised preferentially to the (+) probe compared to those which hybridised preferentially to the (-) probe. Only one of these latter was retained for further study (plasmid pR1). When the filters were dehybridised by NaOH treatment (11) and the probes were reversed for rehybridisation, consistent results were obtained. We concluded that we had isolated colonies harboring plasmids with ds-cDNA inserts representing MCF-7 cell RNAs whose levels are either increased (plasmids pS1-4) or decreased by steroid hormones (plasmid pR1). Confirmation of this conclusion was obtained by hybridising nick-translated plasmids pS1-4 and pR1 to RNA blots carrying equal amounts of cytoplasmic polyA (+) RNA isolated from cells grown either in normal medium [(+) RNA] or in stripped medium plus nafoxidine [(-) RNA]. An example of this analysis using two independent batches of (+) and (-) RNA is shown in Fig. 3 for pS1, pR1, and pN1, a plasmid whose corresponding RNA's level is not affected by oestradiol according to the differential hybridisation study. As expected, pS1 hybridises more strongly to the (+) RNA samples (panel B, lanes 1, 4) , pR1 to the (-) RNA samples (panel C, lanes 2, 3) and pN1 equally strongly to all four samples (panel A). Similar experiments carried out with pS2-4 showed the same result as that obtained with pS1. All four of these plasmids re-

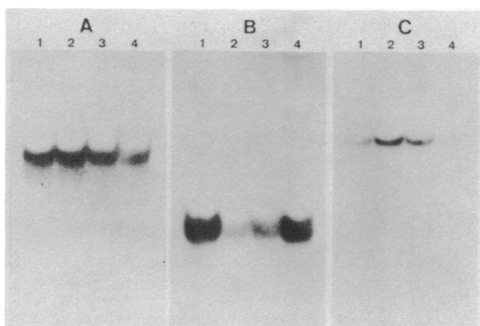

Fig. 3 : Analysis of hormonally-regulated MCF-7 transcripts. MCF-7 cells were maintained in normal medium [(+) samples] or in stripped medium (see text) containing 1 μM nafoxidine [(-) samples]. 2 μg of polyadenylated (+) RNA (lanes 1,4) or (-) RNA (lanes 2,3) from two different experiments were electrophoresed on a 1.5% denaturing formaldehyde agarose gel (13) and transferred to a nitrocellulose filter, before hybridisation to either plasmid pS1 (panel B), pN1 (panel A), or pR1 (panel C) labelled with [^{32}P] by nick-translation (16) (specific activity 10^8 cpm/μg). The same filter was used for all three probes and was dehybridised between different hybridisations (15).

vealed an RNA band of around 600 nucleotides (estimated from electrophoresis in a methylmercury hydroxide gel using an AluI digest of pBR322 as size marker, data not shown) and showed markedly stronger hybridization to (+) rather than (-) RNA. This suggested that they might contain ds-cDNA copies of the same (or a related) RNA, and this possibility was strongly supported by the similarity of the restriction enzyme maps of the four ds-cDNA inserts in pS1-4 (S.Jakowlew, personal communication). Further work was carried out using pS2, which carries the longest insert (around 500 bp).

To demonstrate that oestrogen alone is sufficient to stimulate the level of the RNA represented by pS2, MCF-7 cells were maintained for 10 days in medium supplemented with stripped serum either containing 10 nM oestradiol in 0.2% ethanol or only 0.2% ethanol. Cytoplasmic RNA was prepared from these cells, and electrophoresed together with a sample of RNA prepared from cells grown in normal medium. The RNA was transferred to DBM-paper and hybridised to a [^{32}P]-labelled pS2 probe. It is clear from Fig. 4A that RNA from cells maintained in stripped serum (lane 1) contains much less pS2-related RNA than does RNA from cells maintained in the presence of oestradiol (lane 2) or in normal serum (lane 3), and that addition of oestradiol alone to cells maintained in stripped serum is sufficient to restore the level of pS2 RNA to a level similar to that seen with cells grown in normal medium (compare lanes 2 and 3). The kinetics of accumulation of the pS2 RNA after oestradiol administration were analysed by maintaining MCF-7 cells in stripped medium for 10 days before addition of 10 nM oestradiol for various times. Samples of cytoplasmic RNA were prepared and used to make RNA blots, which were hybridized to a [^{32}P]-labelled pS2 probe. An example of this analysis is shown in Fig. 4B. pS2 RNA reaches a plateau after 24 h exposure to oestradiol, with a definite increase over the basal level 3 h after first exposure.

In summary, we have isolated four ds-cDNA clones representing an MCF-7 RNA whose level is increased rapidly following oestradiol administration to cells deprived of steroid hormones. It is unlikely that the about 600 nucleotide long mRNA corresponding to these clones could code for the 46,000 dalton protein which is excreted in the medium by oestradiol-treated MCF-7 cells (see above). The reason why we did not clone a ds-cDNA corresponding to this protein is unknown, but the actual level of the corresponding mRNA may be very low. Furthermore, it

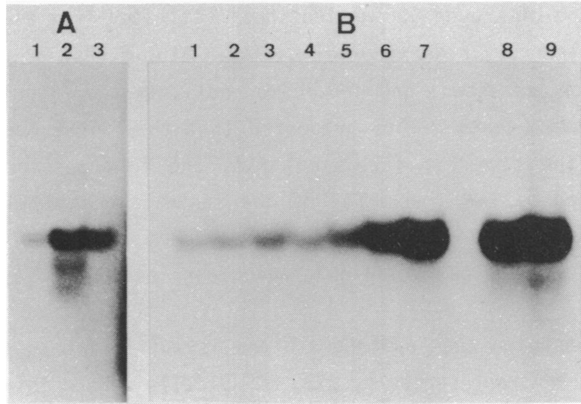

Fig. 4 : Accumulation of an MCF-7 transcript following oestradiol administration. A. On day 1 confluent MCF-7 cells were divided 1:2 and plated in stripped medium [(-)sample] or in the same medium containing 10 nM estradiol [(+) sample]. Every second day until day 10 the cells were washed twice with PBS and re-fed fresh medium. Cytoplasmic RNA was prepared from these cells, together with a sample from cells maintained in 10% fetal calf serum (FCS sample). 10 μg RNA was electrophoresed on a 1.5% agarose gel containing 10 mM methylmercury hydroxide (handled under a fume hood) and transferred to DBM-paper before hybridisation to [^{32}P]-labelled pS2. Lane 1 : (-) RNA Lane 2 : (+) RNA Lane 3 : FCS RNA. B. MCF-7 cells were maintained in medium depleted of steroid hormones as described above for 10 days before treatment for various times with 10 nM oestradiol. Cytoplasmic RNA was prepared and 10 μg electrophoresed, transfered and hybridised as described above. Lanes 1-7 : RNA from cells exposed to oestradiol for 0, 15, 30 min, 1, 3, 8 and 24 h. Lanes 8 and 9: 10 μg cytoplamic and total RNA from cells maintained in 10% FCS, respectively.

has not been demonstrated that the induction of this protein is at the transcriptional level. We have also identified another series of ds-cDNA clones whose corresponding RNAs level seems to be decreased by steroid hormones. Whether the effect is in this case mediated by oestradiol remains to be determined. The availability of pS2 as a cDNA probe for an oestrogen-regulated mRNA in MCF-7 cells has allowed us to show that the corresponding gene is controlled at the transcriptional level (A.M.C. Brown et al., in preparation). We intend to clone the gene from genomic libraries of MCF-7 and normal human DNA, and hope to use such a cloned gene in studies of the DNA sequences required for hormonal induction. We also plan to use pS2 to screen a series of breast cancers for production of the corresponding messenger RNA in an attempt to determine whether expression of this gene is in any way related to the cancerous state.

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