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Identification of Estrogen Regulated Genes in Fe33 Rat Hepatoma Cells by Differential Display Polymerase Chain Reaction and their Hormonal Regulation in Rat Liver and Uterus

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We applied the differential display RT–PCR (ddRT–PCR) technology to identify estrogen-regulated hepatic genes in the estrogen receptor expressing rat hepatoma cell line Fe33. Three genes of known sequences were detected by the ddRT–PCR approach: IGF binding protein-1 (IGFBP-1), vitamin D-dependent calcium-binding protein (CaBP9k) and major acute phase protein (MAP). Effects of ethinyl estradiol on the mRNA levels of these genes were confirmed by "Northern-blot" analysis. If given in combination with dexamethasone and glucagon, ethinyl estradiol caused 40-, 15- and 11-fold increases in the mRNA steady state level of IGFBP-1, CaBP9k and MAP, respectively, in Fe33 cells 24 h after addition of hormone. Besides ethinyl estradiol, the partial estrogen agonist OH-tamoxifen caused dose dependent effects on expression of MAP and IGFBP-1. Estrogen regulation of the respective genes and the modulatory effects of progesterone (10 mg/animal/day) were studied in ovariectomized rats treated subcutaneously for 14 days with 1 μg/animal/day estradiol. "Northern-blot" analysis of liver RNA revealed a 6-fold stimulation of IGFBP-1 mRNA levels in estradiol-treated compared to vehicle-treated rats and a weak but detectable increase of MAP mRNA steady state level (1.6-fold) upon estradiol administration. No effect of estradiol treatment could be monitored for CaBP9k in rat liver. Modulatory effects of progesterone on estradiol-stimulated expression in the liver could be monitored for IGFBP-1 only. In an extension of our investigation on the expression of the three genes in rat liver, we determined their expression and hormonal regulation in the uterus of the same animals. In the uterus, estradiol caused an increase in CaBP9k mRNA. In contrast, IGFBP-1 mRNA levels increased dramatically upon progesterone administration, whereas no effect of estradiol treatment could be detected. MAP mRNA levels increased only after coadministration of estradiol and progesterone. In conclusion, the ddRT-PCR proved to be a powerful method to identify estrogen-regulated genes. The study on the hormonal regulation of three genes stimulated by estrogen in Fe33 cells revealed similarities and differences in their regulation *in vivo* and *in vitro*.

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INTRODUCTION

Estrogen therapy is associated with changes in several liver derived proteins like angiotensinogen [1], coagulation factors [2], steroid hormone binding proteins [3] and proteins involved in lipid metabolism [4]. Specifically, these hepatic effects are caused by estrogens mainly when applied orally. For some hepatic proteins the mechanism of estrogen regulation is indirect and based on modulation of pituitary growth hormone secretion [3]. Several other hepatic genes were shown to underlie direct regulation via the hepatic estrogen receptor (ER) [5]. Aiming to extend our understanding of direct estrogen effects on liver gene expression, we used the ER expressing rat hepatoma cell line Fe33

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which was shown earlier to be suitable for studying estrogen effects on endogenous hepatic genes. Fe33 cells were derived from FTO-2B cells which were stably transfected with the ER [6]. We applied the differential display RT–PCR (ddRT–PCR) method in order to identify hepatic genes being subject to direct estrogen control. The ddRT–PCR was shown recently to be a valuable tool for identification and isolation of cDNAs derived from differentially expressed genes in cells or tissues under different physiological conditions [7, 8].

One aim of this study was to isolate and identify new estrogen regulated genes from Fe33 cells. To verify the results of the ddRT–PCR and to quantify the estrogen effects on the mRNA level of the identified gene products, "Northern" blot analysis was performed. A second aim was to test whether genes underlying estrogen control in Fe33 cells are also subject to estrogen regulation *in vivo*. As a third aim we investigated the effects of sex hormones on expression of the identified genes in the liver in comparison to the uterus as a major target organ of sex hormones. Studies were conducted in ovariectomized rats treated for 14 days with 1 μg/animal/day estradiol and/or 10 mg/animal day progesterone, subcutaneously.

MATERIALS AND METHODS

Hormones

Ethinyl estradiol (EE), 17β-estradiol (E_2), progesterone (P), dexamethasone (Dex) and hydroxy-tamoxifen (OH-tam) were provided by the Medical Chemistry Dept. of Schering AG (Berlin, Germany). Glucagon was obtained from Sigma (München, Germany).

Differential display of mRNA

Differential display was performed as previously described with some modifications [8, 9]. Total cytoplasmatic RNA was extracted from cultured cells according to the guanidinium thiocyanate–phenol–chloroform extraction method of Chomczynski and Sacchi [10]. DNA-free RNA was obtained by treatment with RNAse-free DNAse I (Stratagene GmbH, Heidelberg, Germany) in the presence of placental RNAse inhibitor (Stratagene GmbH, Heidelberg, Germany) for 30 min at 37°C. After phenol–chloroform extraction and ethanol precipitation, four reverse transcriptions were performed using the RNAmap™ differential display system (geneHunter Corporation, Brookline, MA, U.S.A.). Polymerase chain reaction was performed in reaction mixtures containing 0.1 volume of reverse transcription reaction mixture, 1 × PCR buffer, 2 μmol/l each of dGTP, dATP, dTTP and dCTP, 10 μCi 35 S-dATP, 1 μmol/l of the respective T12NX oligonucleotide, 0.2 μmol/l specific arbitrary 10-mer oligonucleotide (Primers AP-1 to AP-5 of the RNAmap™ system were used) and 10 units AmpliTaq™ DNA polymerase (Perkin Elmer Cetus, Almeda, U.S.A.). Amplification was done for 40 cycles with 94°C for 15 s, 40°C for 2 min, 72°C for 30 s and an additional extension period at 72°C for 5 min in a Perkin-Elmer 9600 thermocycler.

3.5 μl of each sample was mixed with 2 μl loading dye and incubated at 80°C for 2 min immediately before loading on a 6% DNA sequencing gel. After electrophoresis, gels were transfered to 3MM paper (Whatman, Maidstone, England), dried and exposed to Kodak XAR-5 film for 24–72 h. Bands differing between the patterns of hormone treated and nontreated cells were identified and the PCR was repeated to confirm the findings.

Band recovery, subcloning and sequence analysis

Bands of interest were cut out and DNA was eluted, soaking the gel slice in 100 μl distilled water for 10 min followed by boiling for 15 min. After ethanol precipitation, the DNA was reamplified by PCR using appropriate primers and PCR conditions as described above, except for the dNTP concentrations of 20 μmol/l and omission of radioisotope. PCR products were identified on a 1.5% agarose gel and subcloned, using the pGEM-T cloning vector (Promega corporation, Madison, Wisconsin, U.S.A.). Double-stranded DNA was sequenced using an ABI Taq Dyedeoxy Cycle Sequencing kit (Applied Biosystems, Foster City, U.S.A.), and the sequences were analysed using the GCG sequence analysis software package.

In vitro studies

FTO-2B cells (rat hepatoma cells, provided by Professor Ryffel, Essen, Germany) were maintained in Dulbecco's minimal essential medium (DMEM, Serva, Heidelberg, Germany) without phenol red, containing 5% charcoal-treated calf serum (FCS, Serva, Heidelberg, Germany) and further supplements as described in results. Fe33 cells (FTO-2B cells stably transfected with an ER expression plasmid by Kaling *et al.* [6], provided by Dr Hilgenfeld, Heidelberg, Germany) were maintained in DMEM-Ham's F12 (1:1, v/v) without phenol red (Gibco/BRL, Berlin, Germany) containing 10% charcoal treated fetal calf serum (FCS) on gelatine-coated 15 cm Petri dishes. When serum free medium was used several supplements were added according to Edmunds *et al.* [11].

Total RNA was isolated from cell cultures using the guanidinium thiocyanate–phenol–chloroform extraction method of Chomczynski and Sacchi [10]. The preparation of polyA$^+$-RNA was carried out according to Medcalf *et al.* [12]. "Northern" blot analysis was performed as described by Sambrook *et al.* [13]. Hybridization was carried out according to a protocol for rapid hybridization (Stratagene, Heidelberg, Germany). For reference hybridization cDNA probes of GAPDH [14] and 1A [15], a cDNA probe coding for subunit I of the cytochrome C oxidase (Lessl *et al.* unpublished) were used. Autoradiography was

performed by exposing Kodak X-omat AR film (Eastman Kodak, Rochester, NY, U.S.A.) to the nylon filters at −80°C in the presence of an enhancing screen. Autoradiograms were analysed by densitometry.

In vivo studies

Animals. Female rats (Wistar, Schering AG; 200 g body weight) were kept in macrolon cages with free access to water and standard rat diet (Altromin) *ad libitum*. The light–dark-cycle was 14:10 h.

Animal treatment. The animals were ovariectomized under ether narcosis 14 days prior to the beginning of the application of the compounds. The rats were treated s.c. for 14 days with either vehicle only [benzyl benzoate/castor oil 4:1 (v/v)], estradiol (E_2) (1 μg/animal/day), progesterone (P) (10 mg/animal/day) or a combination of estradiol (1 μg/animal/day) and, progesterone (10 mg/animal/day) (E_2/P). The animals were killed and desanguinated. Livers and uteri of 7 animals per group were pooled and total RNA was isolated according to the guanidinium thiocyanate–phenol–chloroform extraction method of Chomczynski and Sacchi [10]. The preparation of polyA$^+$-RNA, "Northern" blot analysis and hybridization was performed as described above.

RESULTS

Isolation and identification of differentially expressed mRNAs by ddRT–PCR

To identify estrogen inducible mRNA species, Fe33 rat hepatoma cells were treated for 24 h with 10 nmol/l

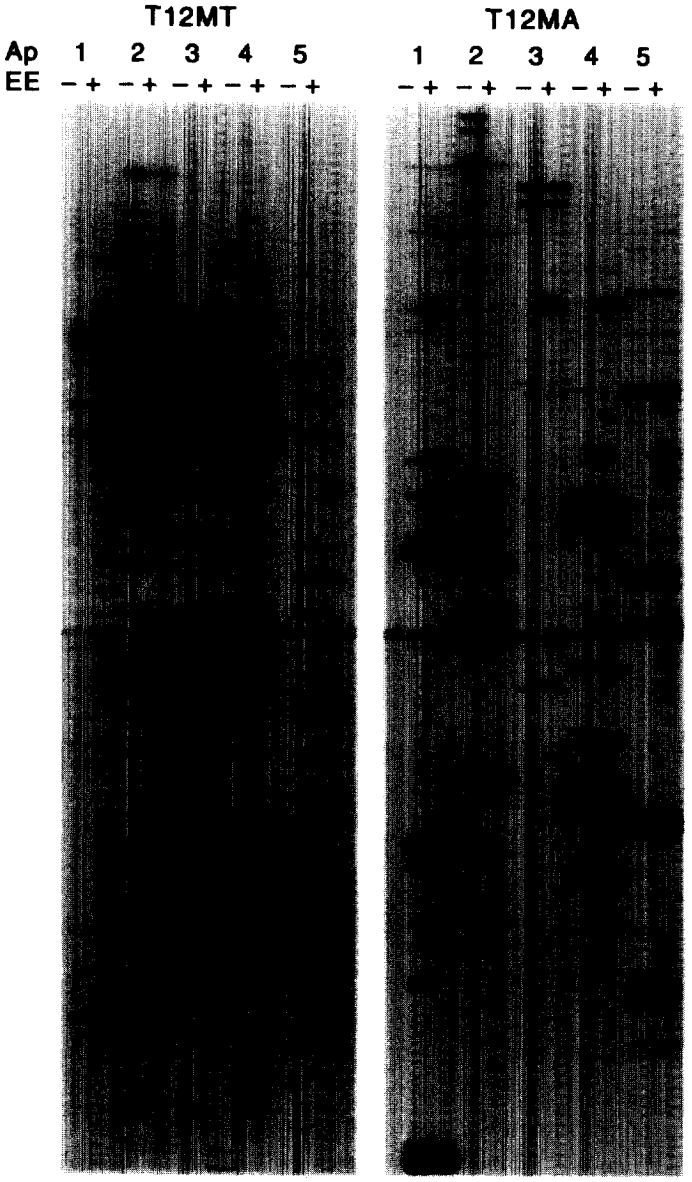

Fig. 1. Pattern of bands corresponding to amplicons obtained from mRNA of hormone treated (+) and nontreated (−) Fe33 cells using the ddRT–PCR technique. T12MT, T12MA and the AP-primers 1–5 of the geneHunter differential display system were used for amplification. The indicated bands correspond to amplicons which were selected for further analysis.

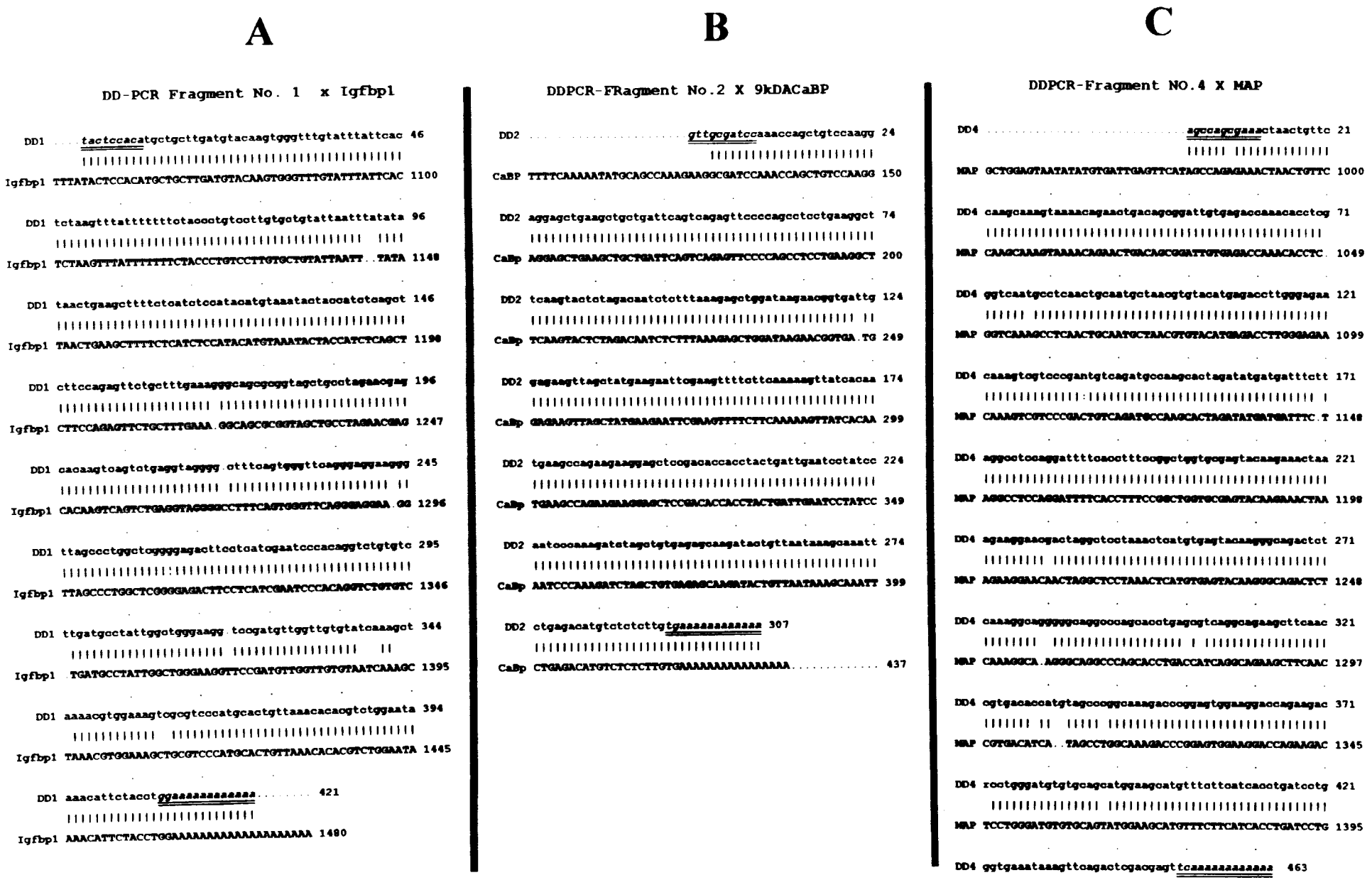

Fig. 2—*legend on facing page.*

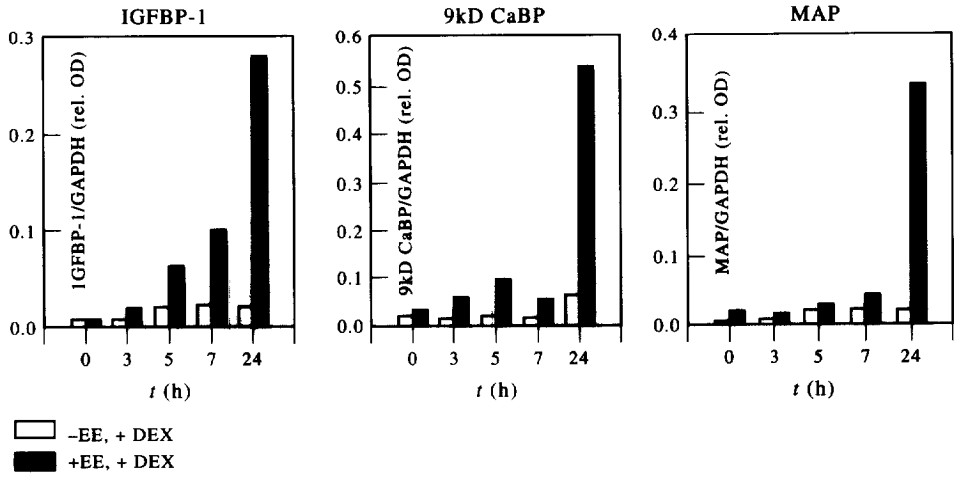

Fig. 3. Time course of changes in the mRNA levels of IGFBP-1, CaBP9k and MAP in Fe33 cells to verify the results obtained by ddRT-PCR. Fe33 cells were cultivated for different times in the presence of dexamethasone/glucagon (Dex/Glu 10^{-7} mol/l), or in the combination of ethinyl estradiol (EE; 10^{-8} mol/l) and dexamethasone/glucagon (Dex/Glu 10^{-7} mol/l). mRNA levels were determined by Northern blot analysis. The data were obtained by laser densitometric analysis of autoradiographies and are presented as relative optical density (re.OD) compared to GAPDH expression.

EE and 100 nmol/l dexamethasone/glucagon (Dex/Glu) [16]. Previous experiments ([16] and our own unpublished results) have shown that stimulation of estrogen responsive genes (angiotensionogen, fibrinogen, CBG) in Fe33 cells may be further increased by cotreatment with Dex/Glu. We, therefore, also cotreated Fe33 cells with EE and Dex/Glu in experiments designed to identify new estrogen-regulated genes by ddRT-PCR.

Combinations of five arbitrary 10-mer primers and four T12NX primers (RNAmap™ differential display system) were used to amplify individual single stranded cDNAs derived from total RNA isolated from estrogen-treated and untreated cells, respectively. Each primer combination displayed 100–200 bands allowing about 2000 different mRNA-species to be screened by this approach. The pattern of amplified cDNAs obtained with the respective primer combinations were highly reproducible (Fig. 1). Comparing the primer-specific patterns of hormone treated and nontreated cells, differences were observed which could be confirmed by at least two repetitions of the ddRT-PCR. For further analysis ten DNA bands with a size of at least 200 bp were chosen that revealed an increased intensity in lanes corresponding to transcripts from estrogen-treated compared to untreated cells. Respective amplicons were isolated from the dried denaturing polyacrylamide gels and were reamplified using the suitable primer sets. The reamplification worked with high efficiency and the cDNA products were consistent in size with the bands in the original display. After subcloning into a pGEM-T plasmid vector the nucleotide sequences of the amplicons were determined. Amplicon 5 turned out to be identical to amplicon 9 and amplicon 7 was identical to amplicon 10. Database analysis revealed the identity of 3 amplicons to sequences present in the EMBL-databank (Fig. 2). Amplicon 1 (compare Fig. 1) had 95% identity to rat insulin-like growth factor binding protein-1 (IGFBP-1), amplicon 2 had 99% identity to rat vitamin D-dependent calcium-binding protein (CaBP9k) and amplicon 4 exhibited 95% identity to rat major acute phase alpha-1 protein (MAP). For the remaining amplicons, no significant homology to any sequence present in the EMBL-databank could be detected.

In vitro studies

Experiment 1 (Fig. 3). In order to test estrogen regulation of the identified genes and to quantify the regulatory effects, Fe33 cells were treated for 3, 5, 7 and 24 h with 10 nmol/l EE in the presence of 100 nmol/l Dex/Glu. "Northern blot" analysis was performed using the cloned amplicons as probes. Three of the five unidentified amplicons did not show detectable signals by "Northern blot" analysis. For the remaining two fragments signals could be detected, but no significant alteration was observed (data not shown). In contrast the level of IGFBP-1, MAP and CaBP9k mRNA showed a clear time dependent regulation by ethinyl estradiol. These results are documented by Fig. 3. The level of IGFBP-1 mRNA increased 40-fold 24 h after

Fig. 2 (opposite). Nucleotide sequence of the clones containing amplicons 1, 2 and 4, respectively, as inserts. Homology of amplicon 1 to the 3′ regions of IGFBP1, amplicon 2 to CaBP9k and amplicon 4 to MAP is shown. Identical residues between the DNA sequences of the amplicons and the respective mRNAs are indicated by vertical lines. The flanking AP and T12MN primers (double underlined) are shown.

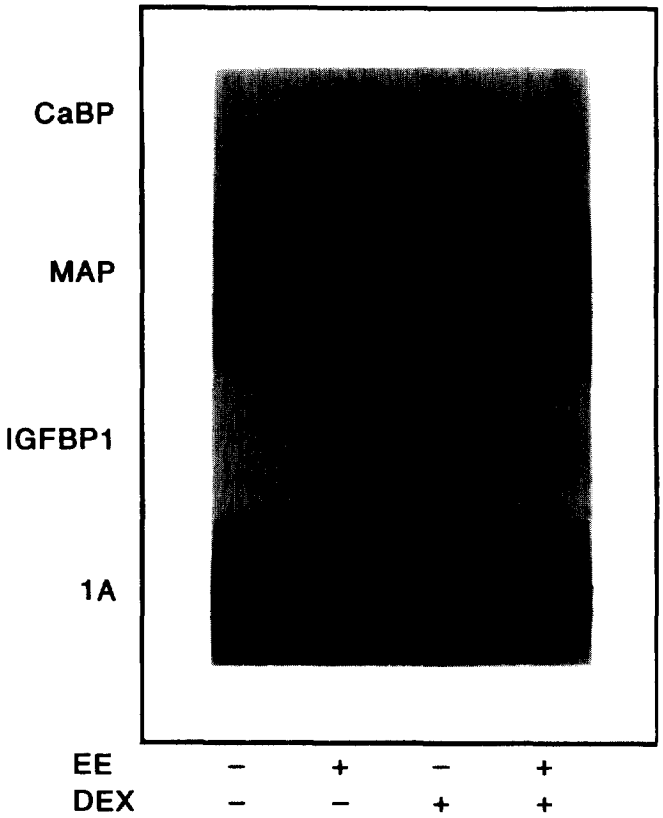

Fig. 4. Northern blot analysis of mRNA levels of IGFBP-1, CaBP9k and MAP in Fe33 cells after cultivation in serum-free medium for 24 h either in the absence of steroid hormones, in the presence of ethinyl estradiol (EE; 10^{-8} mol/l), in the presence of dexamethasone/glucagon (Dex/Glu; 10^{-7} mol/l) or in the presence of a combination of ethinyl estradiol (EE; 10^{-8} mol/l) and dexamethasone/glucagon (Dex/Glu; 10^{-7} mol/l).

EE administration. For CaBP9k a 15-fold and for MAP a 10-fold increase of mRNA-levels could be detected.

Experiment 2 (Fig. 4). The expression of IGFBP-1 and MAP was reported to be regulated by glucocorticoids and glucagon in rat liver [17, 18]. In order to discriminate the effects of glucocorticoids/glucagon and estrogen on IGFBP-1, MAP and CaBP9k expression, Fe33 cells were cultivated for 24 h either in the presence of EE, of Dex/Glu, of EE and Dex/Glu or in the absence of either hormone. The mRNA-levels were determined by "Northern blot" analysis. As to IGFBP-1 and MAP, incubation with either Dex/Glu or EE caused a weak but detectable increase in the mRNA level. Cotreatment with Dex/Glu and EE induced the mRNA-level of IGFBP-1 40-fold and the level of MAP 4-fold, suggesting a synergistic action of both compounds. In contrast the level of CaBP9k mRNA was strongly induced in cells treated with EE (20-fold), whereas coadministration of Dex/Glu and EE caused a somewhat lower induction (15-fold).

In FTO-2B cells, which lack the estrogen receptor, dexamethasone treatment caused similar effects as observed in Fe33 cells. EE treatment was without effect (data not shown).

Experiment 3 (Fig. 5). To further analyse estrogen effects on the three genes mentioned above, a dose–response curve using EE and the partial estrogen agonist OH-tamoxifen (OH-tam), respectively, were administrated to Fe33 cells at increasing concentrations. Cells were grown for 24 h in presence of the compounds. Afterwards cells were harvested, RNA was isolated and analysis of mRNA was carried out by Northern blot analysis. EE and OH-tam caused a dose-dependent increase in mRNA level of IGFBP-1 and MAP. For CaBP9k only a dose-dependent stimulation by EE could be detected. Dose–response curves are shown by Fig. 5.

In vivo studies

Experiment 4 (Figs 6 and 7). To study if the observed effects on expression of IGFBP-1, MAP and CaBP9k in Fe33 cells reflect the *in vivo* situation in rat liver ovariectomized rats were treated subcutaneously for 14 days with E_2. Possible modulatory effects of progesterone were also evaluated by administration of 10 mg progesterone/animal/day either alone or in combination with estradiol. The mRNA levels of IGFBP-1, MAP and CaBP9k were determined by "Northern blot" analysis. The result of this experiment is documented in Fig. 6. For IGFBP-1 and MAP an induction of the mRNA level could be detected. 6- and 1.6-fold increases in mRNA levels were observed for IGFBP-1 and MAP, respectively. CaBP9k mRNA was hardly detectable and no regulatory effect of estrogen treatment could be detected. Progesterone had either no effect (MAP and CaBP9k) or slightly reduced the stimulatory effect of estradiol on liver mRNA levels (IGFBP-1).

In extension of our study on hormonal regulation of expression of the three genes, we investigated their expression in rat uterus in the same animal experiment. Uterine expression of IGFBP-1, MAP and 9 kDa CaBP and effects of estradiol and progesterone treatment were analysed by "Northern blot" analysis (Fig. 7). In the uterus of ovex control rats or in rats treated with estradiol, IGFBP-1 mRNA was not detectable. A dramatic increase was caused by treatment with progesterone either administrated alone or in combination with estradiol. MAP mRNA was detectable in the uterus of estradiol- and progesterone-treated animals only. As expected [20] CaBP9k turned out to be estrogen-regulated in rat uterus whereas progesterone exhibited no effect on expression of this gene.

DISCUSSION

In the present study we identified three genes underlying estrogen regulation in cultured Fe33 rat hepatoma cells. Fe33 cells, a derivative of FTO 2B cells which were stably transfected with the ER[6], were found earlier to be suitable to study estrogen effects on

expression of hepatic genes [16]. Earlier studies also revealed that cotreatment of the cells with estrogen in combination with Dex/Glu caused enhanced stimulation of estrogen sensitive genes like angiotensinogen, fibrinogen and CBG in these cells ([16] and our own unpublished data). We used the same treatment regimen in our attempt to identify new estrogen regulated genes, assuming that the combined treatment of the cells with the three hormones might best reflect the *in vivo* situation. Interestingly we found that two of the genes identified by the present study, IGFBP-1 and major acute phase glycoprotein (MAP), which were thought to be estrogen regulated in Fe33 cells, turned out to also be estrogen-regulated *in vivo* in the liver of ovariectomized (ovex) rats.

ddRT-PCR

For identification of the respective genes we used the differential display PCR method. This recently developed technique [7] has a couple of advantages compared to classical differential expression cloning methods. Differential display RT-PCR offers a straightforward approach to discriminating gene expression under different physiological conditions. One major advantage is its high sensitivity, also allowing the detection of differences in expression of low abundant mRNAs.

In our experiments the ddRT-PCR was performed essentially according to standard protocols [8, 9]. A problem often described connected with the use of this technique is a high rate of 'false positives'. In our approach two of eight selected amplicons turned out to be 'false positives', three genes were not detectable by Northern blot analysis and three genes were proved to be truly estrogen regulated. The undetectable amplicons could be copies of rare transcripts which are not detectable by Northern blot analysis, but it is also possible that they do not originate from mRNA. For example contamination of the RNA with traces of genomic DNA could be the origin of these type of false positive amplicons. To clarify if our undetectable amplicons are false positives or copies of rare transcipts it would be necessary to use more sensitive RNA quantification assays like RNase protection or quantitative PCR.

Regulation of the identified genes by estrogens and progesterone

Out of ten amplicons chosen for further analysis, three were found to exhibit sufficiently high sequence homology to published sequences contained in DNA data banks to allow the identification of corresponding genes.

Amplicon 1 was identified as being derived from IGFBP-1 mRNA. IGFBP-1 belongs to a family of IGF binding proteins that play a role in modulation of biological actions of insulin-like growth factors [20, 21]. IGFBP-1 is synthesized in the liver and is highly

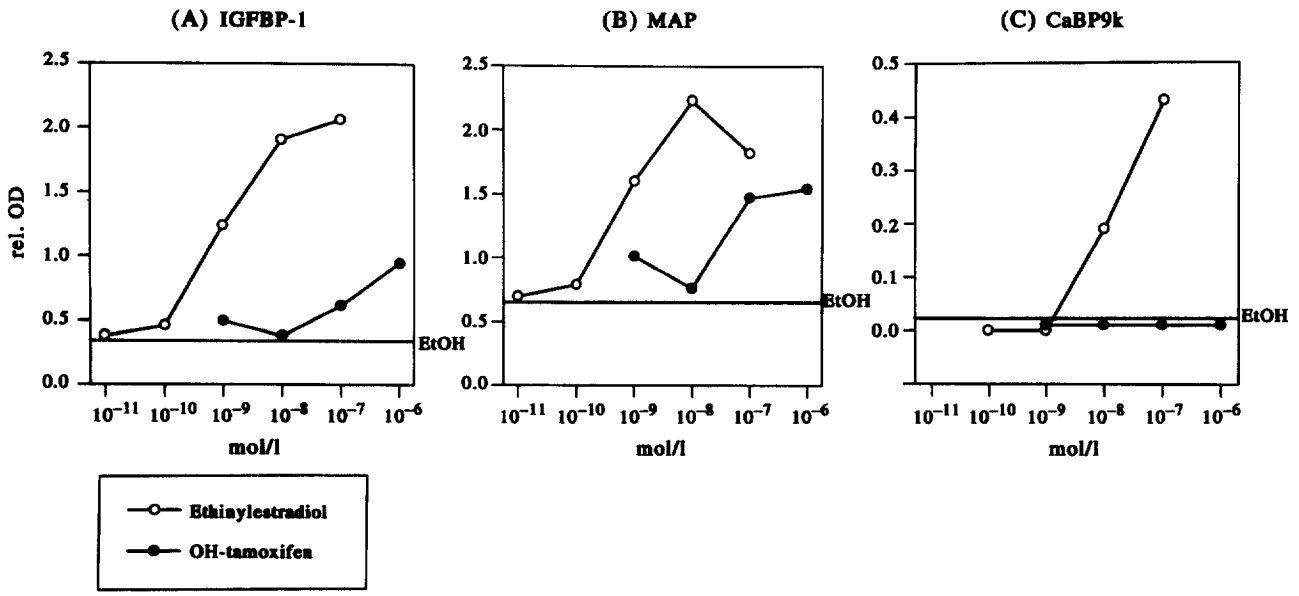

Fig. 5. Dose-dependent stimulation of IGFBP-1 (A), MAP (B) and CaBP9k (C) mRNA levels in Fe33 cells by ethinyl estradiol and the partial agonist OH-tamoxifen. Fe33 cells were cultivated in serum-free medium for 24 h in presence of 10^{-9} to 10^{-7} mol/l EE (○) or 10^{-9} to 10^{-6} mol/l OH-tamoxifen (●), respectively. mRNA levels were determinated by Northern blot analysis. The data were obtained by laser densitometric analysis of autoradiographies and are presented as relative optical density (rel.OD), with signals for GAPDH mRNA being used as a reference. The solid line indicates mRNA levels in cells grown in the absence of hormone. For CaBP9k only a weak but dose-dependent increase in mRNA level in the presence of 10^{-8} and 10^{-7} mol/l EE could be detected. OH-tam exhibited no detectable effect on this parameter.

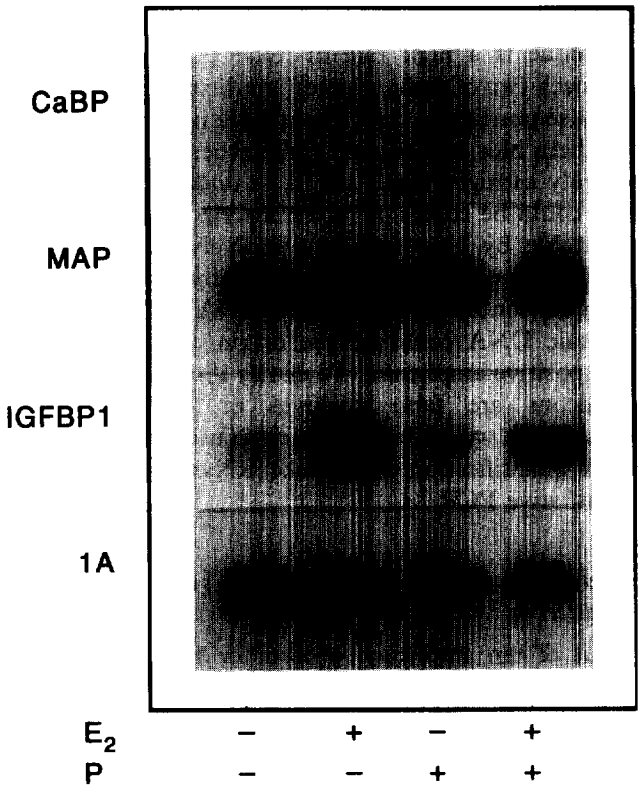

Fig. 6. Northern blot analysis of mRNA levels of IGFBP-1, CaBP9k and MAP in rat liver. Overiectomized rats were treated for 14 days with vehicle only (benzyl benzoate/castor oil = 4:1), estradiol (E2) (1 μg/animal/day), progesterone (P) (10 mg/animal/day) and a combination of E2 and P (E_2/P) (1 μg/animal/day E_2 and 10 mg/animal/day P).

expressed in the decidualized rat uterus [18]. Progesterone regulation of IGFBP-1 was reported for human endometrial stromal cells [22]. Its production in liver was shown to be regulated by GH, insulin, dexamethasone, phorbol esters, catecholamines and glucagon [17, 23–25]. Regulatory cis acting elements in the promotor of the IGFBP-1 gene were described for glucocorticoids, cAMP and phorbol esters [26]. Insulin causes a down regulation of IGFBP-1 gene expression, whereas dexamethasone, cAMP, phorbol ester and catecholamines stimulate IGFBP-1 expression [26]. Very recently, estradiol regulation of IGFBP-1 gene expression in liver and uterus was demonstrated by Molnar and Murphy [27]. Our experiments clearly demonstrate a strong upregulation of IGFBP-1 mRNA by ethinyl estradiol in Fe33 cells and in rat liver by estradiol (Figs 3 and 6). Stimulation of the IGFBP-1 mRNA level by estrogen could also be demonstrated in human hepatoma cells stably transfected with the estrogen receptor (our own unpublished data). Our experiments with Fe33 cells allow us to discriminate the effects of estrogen and dexamethasone/glucagon on the expression of IGFBP-1. Ethinyl estradiol was more effective with regard to stimulation of the IGFBP-1 mRNA level than the combination of dexamethasone and glucagon. Interestingly, we observed that simultaneous administration of dexamethasone, glucagon and ethinyl estradiol had a synergistic effect (Fig. 4) on IGFBP-1 mRNA levels.

We were also able to demonstrate upregulation of IGFBP-1 mRNA by estrogens *in vivo* (Fig. 6). The *in vivo* experiment with ovariectomized rats treated with 1 μg/animal/day estradiol for 14 days revealed a 5-fold increase in liver IGFBP-1 mRNA level. This finding identifies IGFBP-1 as a highly sensitive parameter of hepatic estrogen action. Recent investigations of estrogen effects on angiotensinogen synthesis in rat liver revealed that E_2, in contrast to the metabolically stabilized EE, even at a dose of 3 μg/animal/day had no effect on angiotensinogen serum levels, if applied subcutaneously [16].

Molnar and Murphy [27] very recently reported on an inverse pattern of regulation of hepatic and uterine IGFBP-1 throughout the estrous cycle of the rat, the hepatic IGFBP-1 mRNA level being highest in estrus, the uterine mRNA level being highest in diestrus. Also a transient 2-fold increase in liver IGFBP-1 mRNA was observed upon single subcutaneous administration of 10 μg/kg estradiol. Maximal stimulation was already attained 1 h after estradiol treatment. Our study shows that long term estradiol treatment leads to more pronounced effects on IGFBP-1 mRNA levels (about a 5-fold increase in liver IGFBP-1 mRNA level).

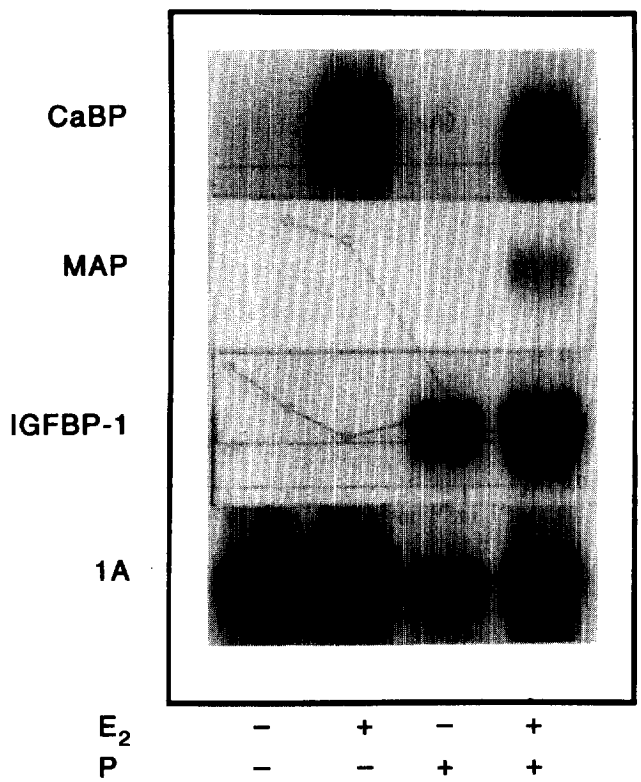

Fig. 7. Northern blot analysis of IGFBP-1, CaBP9k and MAP mRNA levels in rat uterus. Overiectomized rats were treated for 14 days with vehicle only (benzyl benzoate/castor oil = 4:1), estradiol (E2) (1 μg/animal/day), progesterone (P) (10 mg/animal/day) and a combination of E2 and P (E_2/P) (1 μg/animal/day E_2 and 10 mg/animal/day P).

Progesterone, if given in combination with E_2, counteracted the stimulatory effect of E_2 on IGFBP-1 mRNA levels in ovex rats. A direct negative effect via the hepatic progesterone receptor (PR) can be excluded as a possible explanation for the observed interference of progesterone with the E_2-stimulated increase in IGFBP-1 mRNA level. PRemRNA and protein were not detectable in rat liver by RT–PCR or ligand binding studies, respectively (our own unpublished studies). Therefore, unknown indirect mechanisms are assumed to be responsible for the observed effect.

The physiological relevance of the increase in liver IGFBP-1 production upon estrogen administration is difficult to interpret. It may be related to the known effects of estrogen treatment on synthesis of IGF-I in the liver. We and others have demonstrated that estrogen treatment of rats leads to a significant decrease of the level of liver IGF-I mRNA (40%) [16, 27]. Under the same conditions a 50% decrease of IGF-I plasma levels could be observed. The increase in IGFBP-1 production and a reduction in IGF-I synthesis may represent synergistic mechanisms to reduce the level of biologically active IGF-I.

By the *in vivo* study with ovex rats we were able to confirm the suggested regulatory effect of progesterone on uterine expression of IGFBP-1 [22] (Fig. 7). Expression of IGFBP-1 was hardly detectable in ovex rats and in ovex rats treated with estradiol (1 μg/animal/day for 14 days). Progesterone and a combination of progesterone and estradiol caused a dramatic increase in uterine IGFBP-1 mRNA level.

Amplicon 4 was identified as major acute phase protein (MAP) which was shown to be identical to T-kininogen [28, 29]. Another protein previously reported to be identical to MAP/T-kininogen is cysteine proteinase inhibitor [29, 30]. T-kininogen is a component of the kininogen–kallikrein–kinin system that mediates events occuring during the inflammatory response [30–32]. As part of the acute phase response to tissue injury or infection, a dramatic increase in MAP mRNA level in the liver is found, the concentration amounting to up to 1% of total liver RNA after induction of acute inflammation. Expression of MAP in the liver during acute phase response is stimulated by interleukin-6. A consensus sequence of the induction of acute phase protein gene transcription by interleukin-6 was monitored in the rat T-kininogen gene promotor [31, 32]. Like other acute phase response genes the promotor of MAP contains a glucocorticoid response element, and activation of the gene *in vitro* by glucocorticoid was reported [32, 33]. So far, estrogen regulation of this gene had not been described. In our experiments MAP was shown to be estrogen regulated *in vitro* and *in vivo* (Figs 3–6), the pattern of its regulation being analogous to that observed for IGFBP-1 and angiotensinogen [16]. In Fe33 hepatoma cells, ethinyl estradiol and dexamethasone exhibited an additive stimulatory effect on MAP mRNA level (Fig. 4), however the level of expression of this gene was low if compared to IGFBP-1. *In vivo*, a clear increase in MAP mRNA level could be demonstrated, the induction of MAP mRNA being lower than observed for IGFBP-1 (Fig. 6). Estrogen regulation of other acute phase protein genes has been reported [16]. Stimulation of fibrinogen subunit mRNAs (our own unpublished data) and of angiotensinogen mRNA by estrogen in Fe33 cells was demonstrated [16]. An estrogen responsive cis element was described for the rat angiotensinogen gene [34].

Amplicon 2 could be identified as a vitamin D-dependent calcium-binding protein (CaBP9k, Calbindin-D9k). This calcium binding protein is suggested to play an important role in calcium transport and resorption, respectively, in diverse tissues like bone and cartilage [35], intestine and kidney [36], uterus and placenta [19, 37, 38]. Its expression in mineralized tissue and in intestine and kidney is induced by calcitriol. In rat uterus, its synthesis is estrogen dependent in uterine stroma and smooth muscle tissue. In uterine epithelium it is highly expressed during pregnancy [37, 38]. Estrogen effects on expression of uterine CaBP9k gene are due to direct transcriptional regulation. The promotor of the CaBP9k gene comprizes an imperfect palindromic estrogen-responsive element which confers estrogen receptor binding and estrogen-dependent transcriptional activity [19].

Our data obtained in Fe33 cells demonstrate a clear increase of the mRNA level of CaBP9k after treatment with estrogens (Figs 3 and 4). In contrast to IGFBP-1 and MAP, no synergistic effect of EE/dexamethasone treatment could be observed. CaBP9k mRNA shows the strongest upregulation by estrogen treatment, whereas dexamethasone instead exhibited a negative effect on the expression of this gene. In our *in vivo* experiments, CaBP9k was hardly detectable in rat liver and no regulation by estradiol could be demonstrated. Regulation of CaBP9k by estrogens in liver cells might be specific for Fe33 hepatoma cells which is not reflected by the *in vivo* situation. Because of the low level of expression of CaBP9k mRNA in rat liver, its physiological function in this tissue is unclear.

In summary, our data demonstrate that the ddRT–PCR technology is a powerful method for the identification of differentially expressed genes. Fe33 cells again proved to be suitable to study estrogen effects on hepatic gene expression. IGFBP-1 and MAP could be shown to be regulated by estrogens *in vitro* and *in vivo*. Interestingly, ethinyl estradiol and dexamethasone exhibited a synergistic effect on these two liver proteins in Fe33 cells. IGFBP-1 showed the most pronounced reaction upon estrogen treatment in Fe33 cells and also in rats, indicating its potential of being applied as a sensitive marker for hepatic estrogen action *in vitro* and *in vivo*. In the uterus, IGFBP-1 expression is not stimulated by estradiol, whereas progesterone

was found to be a potent inducer of its mRNA level. Similarly MAP was found to be progesterone-regulated in rat uterus. CaBP9k was found to be estrogen regulated in Fe33 rat hepatoma cells and in rat uterus but not in rat liver.

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