Identification and Characterization of Estrogen-regulated RNAs in Human Breast Cancer Cells*

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Two cDNA libraries have been constructed with RNA prepared from the estrogen-responsive breast cancer cell lines, MCF7 and ZR 75. They were screened by differential hybridization for estrogen-regulated sequences. A total of 11 different RNAs were isolated from the MCF7 cell cDNA library and four from the ZR 75 cell cDNA library. Only two sequences were isolated from both libraries. The levels of the 13 different RNAs are induced between 2.5- and 100-fold by estrogen in MCF7 cells.

The expression and regulation by estrogen of the RNAs was examined in eight different human tumor cell lines. The relative abundance of each RNA varied in the different cell lines. The expression of three RNAs (pNR-1, pNR-2, and pNR-25) was detected only in estrogen-responsive breast cancer cells. The sequences that were expressed in all eight cell lines were regulated by estrogen only in the three estrogen-responsive breast cancer cell lines.

The response of the RNAs to other classes of steroids and to different concentrations of estrogen was characterized in more detail. The extent to which different concentrations of estradiol induced each RNA varied, but half-maximal induction of most of the RNAs occurred between 2 and $5 \times 10^{-11}$ M. The time at which increased RNA levels were first detected following exposure to estradiol also varied. Estrogen increased the levels of some RNAs within 15 min, while for others there was a lag of 4 h.

It has been known for almost 100 years that breast cancer can be estrogen responsive. Endocrine ablation therapy, such as ovariectomy, causes remission in approximately 30% of premenopausal women with breast cancer. Additive therapies involving the antiestrogen tamoxifen or aminoglutethimide also cause remission in about one-third of breast cancer patients.

Steroids are thought to act by altering the expression of a limited number of responsive genes. The effects of estrogens are mediated by a nuclear, transacting, transcription-regulator protein of 67 kDa called the estrogen receptor. The mechanisms by which estrogens increase transcription are unclear but are thought to include an interaction of the steroid-receptor complex with specific DNA sequences within or around responsive genes. Approximately 60% of breast tumors contain detectable levels of estrogen receptor, but only half the patients with estrogen receptor positive tumors benefit from tamoxifen therapy. Estrogens induce the progesterone receptor in human breast cancer cells (1), and measurement of the two steroid receptors may improve prediction of response to antiestrogen therapy.

Recently, several breast cancer cell lines have been established in culture, mainly from metastatic cells (e.g. 2). While most of these cells do not contain detectable levels of the estrogen receptor, others do (3, 4) and their proliferation is stimulated by estrogen treatment (5–7).

The identification of estrogen-regulated mRNAs in breast cancer cell lines would be of value in several respects. First, availability of cloned probes for estrogen-responsive genes in such a system should facilitate the unraveling of the molecular mechanisms involved in estrogen action. Second, they would provide specific responses with which to characterize the action of antiestrogens in their target cells. Third, the encoded proteins may provide clinically useful markers of estrogen-responsive breast cancer. Finally, as estrogens are thought to be involved in the etiology and proliferation of human breast cancer cells, inducible genes might be identified that are involved in regulating the proliferation of breast cancer cells or the transformation of normal breast epithelial cells.

Recently, three estrogen-regulated RNAs have been isolated from MCF7 breast cancer cells by ourselves (8, 9) and others (10, 11). We now report the results of screening two large cDNA libraries, constructed with RNA from MCF7 and ZR 75 cells, by differential hybridization. Thirteen different estrogen-regulated sequences were isolated. Their RNA levels were increased between 2.5- and 100-fold by estradiol.

**MATERIALS AND METHODS**

**Cell Culture—**MCF7, T47D (12), ZR 75 (13), MDA MB231 (14), BT 20 (15), HBL 100 (16), Hela (17), and A431 (16) cells were maintained in Dulbecco’s modified Eagle’s medium containing 5–15% fetal calf serum and 1 μg/ml insulin. For hormonal treatments, cells were plated into T25 or T75 flasks and grown to confluence. They were then withdrawn from the steroids present in the routine culture medium by culture for 5–7 days in phenol red-free modified Eagle’s medium containing 10% newborn calf serum, treated with dextran-coated charcoal to remove endogenous steroids, and 1 μg/ml insulin. During the first 3 days of culture, the cells were washed twice prior to the medium change. Withdrawn cells were then cultured continuously in the above medium, whereas treated cells were cultured in the above medium supplemented with the indicated hormone. During withdrawal and treatment, culture medium was changed daily.

**Preparation of cDNA Library—**The MCF7 and ZR 75 cell cDNA libraries were constructed from poly(A)* RNA of cells grown continuously in the presence of 10% fetal calf serum and 1 μg/ml insulin as described elsewhere (8). The cDNA was synthesized with reverse transcriptase and *Escherichia coli* DNA polymerase. It was treated with S1 nuclease, size selected by fractionation on Sephadex S200, stained with 20%, and annealed with dG-tailed, KpnI-digested pUC19. JM 105 cells were transformed with the annealed DNA and colonies containing recombinant plasmids identified by plating on agar containing isopropyl-1-thio-β-D-galactopyranoside and X-Gal. Individual colonies were inoculated into the wells of microtiter dishes.
and screened by differential hybridization as described previously (8). Colonies from the MCF; library were grown on nitrocellulose whereas colonies from the ZR 75 library were grown on nylon (Pall Biodyne).

Preparation and Analysis of RNA and DNA—Plasmid DNA was prepared from the recombinants by alkaline lysis, purified by phenol chloroform extraction and ethanol precipitation, and banded on cesium chloride gradients. The cDNA inserts were characterized by restriction enzyme analysis. DNA (10 ng) of the recombinants in pUC 19 was immobilized as a matrix of dots on nitrocellulose as described previously (19).

RNA was prepared, denatured, electrophoresed, and transferred to nitrocellulose or nylon membranes as described previously (8).

Labeling of RNA Probes and Hybridization—cDNA sequences were subcloned into the commercially available vectors, pGEM and bluescript. The multiple cloning sites contained within these vectors are flanked by promoters for T7, T3, or SP6 RNA polymerases. Labeled probes were synthesized by transcription and then hybridized to the immobilized RNA at 65 °C, or to the immobilized DNA at 55 °C, in solutions containing 50% formamide (20). The amount of radiolabeled probe hybridized was quantified by densitometric scanning of the autoradiographs.

RESULTS

Isolation of Estrogen-regulated RNAs—cDNA libraries were constructed from the mRNA of the MCF; and ZR 75 estrogen-responsive breast cancer cell lines. To identify estrogen-regulated sequences, 15,000 individual colonies from each library were screened by differential hybridization. Labeled cDNA was synthesized from poly(A)+ RNA prepared, either from cells that had been cultured in the absence of estrogens, or from cells first withdrawn from steroids and subsequently cultured in the presence of estradiol. Colonies that gave hybridization signals of different intensity with the two probes were put through a second round of differential hybridization.

Plasmid DNA was prepared from all colonies that again appeared to contain estrogen-regulated sequences. The DNA was labeled by nick translation and hybridized to Northern transfers of total RNA prepared from cells withdrawn from steroids and from cells withdrawn and then treated with estradiol. Recombinants that hybridized to discrete RNA species that were induced more than 2.5-fold were taken to contain estrogen-regulated RNA sequences.

The results of screening both cDNA libraries are summarized in Table I. A large number of false positives were taken through to the second round. Of the 154 recombinants in the third rounds, 30% were either false positives or were regulated less than 2.5-fold by estrogen. In total 62 recombinants containing estrogen-regulated sequences were isolated from the MCF; library and 45 from the ZR 75 library.

Many of the recombinants hybridized to RNAs of the same size and abundance that were induced to the same extent by estradiol. One example of each RNA from both the libraries is shown in Fig. 1. We isolated 11 different regulated RNAs from the MCF; cell cDNA library and four from the ZR 75 cell cDNA library. With the exception of the probes for pNR-1 and pNR-25, the recombinants hybridized to a single RNA. The RNAs varied in size, from between 600 bases for the pNR-2 and pNR-105 to about 9 kilobases for the large pNR-25 RNA. The fold induction of the different RNAs by estradiol also varied, from 2.5-fold for the pNR-7 RNA to over 100-fold for the pNR-25 RNA.

Identification of Recombinants Containing the Same RNA—The RNAs varied in abundance by approximately two orders of magnitude. Some of them, such as the pNR-101 RNA, could easily be measured using nick-translated probes, while several others were more difficult to detect. For instance the levels of the pNR-13, pNR-20, and pNR-25 RNAs could not be measured with nick-translated probes in total RNA prepared from cells cultured in the absence of estradiol. It was therefore difficult to measure the degree of regulation of some of the RNAs by Northern analysis.

Plasmid DNA was separated by gel electrophoresis and transferred to nitrocellulose. Plasmid DNA was isolated, labeled by nick translation, and hybridized to the filters. The lengths of the RNAs hybridizing are shown on the right in nucleotides.

| TABLE I |
| Results of screening the MCF; and ZR 75 cDNA libraries for estrogen-regulated sequences, by differential hybridization |
| No. of recombinants analyzed | MCF; | ZR 75 |
| First round (colony hybridization) | 15,000 | 15,000 |
| Second round (colony hybridization) | 460 | 317 |
| Third round (Northern analysis) | 93 | 61 |
| Regulated (>2.5-fold) | 62 | 45 |

FIG. 1. Profiles of estrogen-regulated mRNAs. Total RNA prepared from withdrawn (C) and estradiol-treated (E2) MCF; or ZR 75 cells was separated by gel electrophoresis and transferred to nitrocellulose. Plasmid DNA was isolated, labeled by nick translation, and hybridized to the filters. The lengths of the RNAs hybridizing are shown on the right in nucleotides.

1 and pNR-25, the recombinants hybridized to a single RNA. The RNAs varied in size, from between 600 bases for the pNR-2 and pNR-105 to about 9 kilobases for the large pNR-25 RNA. The fold induction of the different RNAs by estradiol also varied, from 2.5-fold for the pNR-7 RNA to over 100-fold for the pNR-25 RNA.

Subcloning the cDNA Inserts—The RNAs varied in abundance by approximately two orders of magnitude. Some of them, such as the pNR-101 RNA, could easily be measured using nick-translated probes, while several others were more difficult to detect. For instance the levels of the pNR-13, pNR-20, and pNR-25 RNAs could not be measured with nick-translated probes in total RNA prepared from cells cultured in the absence of estradiol. It was therefore difficult to measure the degree of regulation of some of the RNAs by Northern analysis.

cDNA corresponding to each RNA was therefore subcloned into vectors that permit transcription of RNA from phage promoters flanking the cDNA insert. The increased sensitivity of hybridization obtained with these probes allowed us to determine more precisely their degree of regulation by estrogen.
probes, for instance pNR-17, only hybridized to their own pUC 19 recombinant. A few, as predicted from the Northern transfer and restriction enzyme analyses, cross-hybridized. Two RNAs had been isolated from both cell lines. The pNR-2 and pNR-105 recombinants contained the same RNA sequence. In addition the pNR-8 probe isolated from the MCF7 cDNA library cross-hybridized with the pNR-102 cDNA isolated from the ZR 75 library. Although two recombinants could contain nonoverlapping cDNA from the same regulated RNA, the size, abundance, distribution (see below), and degree of estrogen regulation of the different RNAs rules out this possibility.

A summary of the RNAs isolated by differential screening of each cDNA library is given in Table II. Of the 11 sequences isolated from the MCF7 cells, three were isolated more than once. For instance the pNR-2 RNA that is relatively abundant and dramatically induced by estrogen was isolated 47 times. The pNR-1 RNA is very sensitive to estrogen but less abundant, whereas the pNR-7 RNA is only induced 2.5-fold by estrogen but is more abundant.

Only four estrogen-inducible RNAs were isolated from the ZR 75 library, and two of these were also isolated from the MCF7 library. We have therefore identified a total of 13 different estrogen-regulated sequences.

Distribution of the Estrogen-regulated Sequences—The 13 probes were hybridized to RNA prepared from eight different human cell lines cultured in medium containing 10% fetal calf serum. This medium contains physiological concentrations of estrogen. The MCF7, T47D and ZR 75 cell lines were derived from metastatic breast cancer cells and are estrogen responsive. The MDA MB231 cell line was also derived from metastatic breast cancer cells whereas the BT 20 cell line was established from a primary breast tumor. These two cell lines are not thought to be estrogen responsive. The Hela cell line was derived from a cervical carcinoma and the A431 cell line from a vulval carcinoma.

Three examples of the different results obtained are shown in Fig. 2. The pNR-1 RNA was only detected in the three estrogen-responsive breast cancer cell lines. It was most abundant in the MCF7 cell line and least abundant in the ZR 75 cell line. The pNR-8/102 RNA was present in all the cell lines tested. It was most abundant in the MCF7 and ZR 75 cells. The pNR-17 RNA had an unusual distribution. It was detected in MCF7, MDA MB231, and Hela cells, and following a longer exposure in ZR 75 and T47D cells.

The relative abundance of the RNAs in the eight cell lines, expressed for each RNA as a percentage of its most abundant level, is shown in Table III. Three RNAs, pNR-1, pNR-2, and pNR-25, were only detected in the three estrogen-responsive breast cancer cell lines. Nine RNAs were expressed in all the eight cell lines tested. The relative abundance in the different cell lines was different for each RNA. The pNR-17 RNA was unusual in that it was not expressed in all cell lines, but its expression was not restricted to estrogen-responsive cells.

Induction by Estrogen in the Different Cell Lines—The effect of estrogen on the different RNAs was tested in the eight cell lines. The cells were first withdrawn and then cultured for 3 days in the withdrawal medium alone or in the withdrawal medium containing 10^-8 M estradiol. Total RNA was prepared and the RNA levels measured as described under "Materials and Methods." The relative abundance of the RNAs in cells cultured under these conditions was not the same as in cells cultured in 10% fetal calf serum. Three examples of the results obtained are shown in Fig. 3. The pNR-25 RNA is induced by estradiol in the three estrogen-responsive cell lines in which it is expressed. The pNR-100 RNA is expressed in the eight cell lines tested, but its level is only increased by estradiol in the three estrogen-responsive breast cancer cell lines. While the fold induction of the pNR-23 RNA by estradiol is much lower, it is also induced in the three estrogen-responsive cell lines.

The fold induction by estradiol of the 13 estrogen-regulated RNAs, in the eight cell lines is shown in Table IV. The pNR-1, pNR-2, and pNR-25 RNAs were all induced by estradiol in the three estrogen-responsive cell lines in which they are expressed. In general the other RNAs were only induced by estrogen in the three estrogen-responsive breast cancer cell lines. For each RNA the extent of induction varied in the different cell lines. For instance the pNR-100 RNA was less induced in the T47D cell line whereas, the pNR-7 RNA was most induced in this cell line. The exception is the pNR-13 RNA which was induced 5-fold by estradiol in A431 cells. The regulation of the pNR-17 RNA followed the general pattern described above, but we were unable to quantify its level in withdrawn T47D cells.

Concentration Dependence of the Induction by Estradiol—The induction of the different RNAs was characterized in more detail by measuring their response to different concen-
The abundance of each RNA is expressed as a percentage of its highest level. The values are a mean of two (A431 and BT20), three (T47D, ZR 75, MDA MB231, HBL100, and HeLa) or four (MCF7) experiments.

| RNA   | MCF7 | T47D | ZR 75 | MDA-MB231 | BT 20 | HBL 100 | HeLa | A431 |
|-------|------|------|-------|------------|-------|---------|------|------|
| pNR-1 | 100  | 5    | 5     | 2          | 25    | 15      | 5    | 20   |
| pNR-2/105 | 100   | 3.5  | 30   | 10         | 10    | 30      | 20   | 15   |
| pNR-7 | 100  | 35   | 30   | 35         | 10    | 30      | 20   | 60   |
| pNR-8/102 | 100   | 30   | 60   | 15         | 5     | 15      | 100  | 60   |
| pNR-13 | 60   | 100  | 15   | 15         | 10    | 30      | 80   | 15   |
| pNR-17 | 50   | 1    | 15   | 5          | 10    | 15      | 100  | 30   |
| pNR-20 | 100  | 10   | 15   | 35         | 10    | 35      | 25   | 80   |
| pNR-21 | 55   | 50   | 70   | 100        | 10    | 10      | 80   | 40   |
| pNR-22 | 35   | 15   | 15   | 80         | 15    | 65      | 45   | 100  |
| pNR-23 | 30   | 40   | 35   | 60         | 40    | 100     | 30   | 70   |
| pNR-25 | 60   | 70   | 100  | 15         | 20    | 15      | 8    | 35   |
| pNR-100 | 50  | 17   | 100  | 15         | 30    | 45      | 45   | 15   |

![Relative abundance of the regulated RNAs in different human cancer cell lines](image)

Fig. 3. Regulation of pNR-25, pNR-23, and pNR-100 RNAs by estradiol in different cell lines. The cells were withdrawn for 5 days and grown in the presence of 10^{-8} M estradiol for 2 days. Total RNA was prepared, separated by gel electrophoresis, and then transferred to a nylon membrane. The filters were hybridized with 32P-labeled probe as described under "Materials and Methods."

The fold induction by estradiol and the concentration of estrogen required to achieve half-maximal induction of the 13 RNAs is shown in Table V. The inductions varied from 2- to 100-fold. Half-maximal induction occurred over a range of estradiol concentrations from 2 \times 10^{-11} M for the pNR-25 RNA to >10^{-10} M for the pNR-25 RNA. The majority of the RNAs were half-maximally induced by estradiol between 2 and 5 \times 10^{-11} M.

**Time Course of the Induction by Estradiol**—The time required for estradiol to induce the RNA levels was determined using 10^{-8} M estradiol. The cells were withdrawn from steroids and then treated with estradiol for various lengths of time. Total RNA was prepared, and the levels of the different RNAs were measured as described under "Materials and Methods." The time courses of the induction of pNR-17, pNR-25, and pNR-100 are shown in Fig. 5. There was no detectable lag in the induction of the pNR-17 RNA by estradiol. Its RNA level was higher in cells cultured for 15 min in the presence of estradiol than in the withdrawn cells. The induction of this RNA was maximal after 24 h of estradiol treatment. In contrast there was a lag in the induction of the pNR-25 RNAs by estradiol. An increase in RNA levels was first detected after 4 h of estradiol treatment, and the lag was longer for the 1-kilobase RNA than for the 9.5-kilobase RNA. An increase in the pNR-100 RNA level was first detected in cells cultured in the presence of estradiol for 2 h.

The results obtained for these and other RNAs are summarized in Table VI. For three RNAs there was no detectable

| RNA | MCF7 | T47D | ZR 75 | MDA-MB231 | BT 20 | HBL 100 | HeLa | A431 |
|-----|------|------|-------|------------|-------|---------|------|------|
| pNR-1 | 12   | 5    | 8     | 2          | 1     | 1       | 1    | 1    |
| pNR-2/105 | 100   | 30   | 40   | 1          | 1     | 1       | 1    | 1    |
| pNR-7 | 2    | 6    | 1.5   | 2          | 1     | 1       | 1    | 1    |
| pNR-8/102 | 3    | 2.5  | 2.0   | 1          | 1     | 1       | 1    | 1    |
| pNR-13 | 8    | 3    | 2.5   | 1          | 1     | 1       | 1    | 1    |
| pNR-17 | 5    | ~2   | 2     | 1          | 1     | 1       | 1    | 1    |
| pNR-20 | 7    | 1.5  | 1.5   | 1          | 1     | 1       | 1    | 1    |
| pNR-21 | 3.5  | 2.5  | 2.5   | 1          | 1     | 1       | 1    | 1    |
| pNR-22 | 5.5  | 3    | 2     | 1          | 1     | 1       | 1    | 1    |
| pNR-23 | 2.5  | 2    | 3     | 1          | 1.5   | 1       | 1    | 1    |
| pNR-25 | 100  | 17   | 50    | 1          | 1     | 1       | 1    | 1    |
| pNR-100 | 30   | 5    | 15    | 1          | 1     | 1       | 1    | 1    |
| pNR-101 | 4    | 2    | 2     | 1          | 1     | 1       | 1    | 1    |
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FIG. 4. Effects of different concentrations of estradiol on pNR-1 and pNR-25 RNA levels. MCF7 cells were withdrawn for 5 days and then grown in the presence of a range of estradiol concentrations between $10^{-13}$ and $10^{-9}$ M. Total RNA was prepared and 10 μg was separated by gel electrophoresis and then transferred to a nylon membrane. The filters were hybridized as described under "Materials and Methods." Autoradiographs of the hybridization with pNR-1 (A) and pNR-25 (B) are shown.

TABLE V
Sensitivity of the regulated RNAs to estradiol
The values are the mean of three experiments. Standard errors were less than 20% of the mean value except for pNR-23 which was 26% of the mean.

| RNA      | Induction by estradiol |
|----------|------------------------|
|          | 1/2 Maximum | -Fold |
| pNR-1    | $10^{-11}$  | 10    |
| pNR-2/105| $5 \times 10^{-11}$ | 100   |
| pNR-7    | $2 \times 10^{-11}$ | 2     |
| pNR-8/102| $3 \times 10^{-11}$ | 3     |
| pNR-13   | $3 \times 10^{-11}$ | 8     |
| pNR-17   | $3 \times 10^{-11}$ | 6     |
| pNR-20   | $2 \times 10^{-11}$ | 7     |
| pNR-21   | $3 \times 10^{-11}$ | 3.5   |
| pNR-22   | $3 \times 10^{-11}$ | 5.5   |
| pNR-23   | $2 \times 10^{-12}$ | 2.5   |
| pNR-25   | $7 \times 10^{-10}$ | 70    |
| pNR-100  | $5 \times 10^{-11}$ | 30    |
| pNR-101  | $10^{-11}$  | 4     |

FIG. 5. Time course of the induction of the pNR-17, pNR-25, and pNR-100 RNAs by estradiol. MCF7 and ZR 75 cells were withdrawn and then cultured for the indicated lengths of time in the withdrawal medium alone (○—○) or containing $10^{-8}$ M estradiol (●—●). Total RNA was prepared and analyzed as described under "Materials and Methods." The amount of radiolabeled probe hybridized is plotted as a percentage of the maximum value for each RNA.

TABLE VI
Time required by estradiol to induce the different regulated RNAs
Values are the mean of two experiments. The individual values varied by less than 20% of the mean.

| RNA  | Lag | 1/2 Maximum | -Fold |
|------|-----|--------------|-------|
| pNR-1|     | 6            | 12    |
| pNR-2/105| 1 | 11           | 95    |
| pNR-13| 4  | 6            | 3     |
| pNR-17| 4  | 9            | 5     |
| pNR-21| 6  | 2.5          |       |
| pNR-25| 4  | 12           | 150   |
| pNR-100| 2 | 16           | 10    |

DISCUSSION
Different Sequences Isolated from Two Libraries—We have isolated 11 different estrogen-regulated RNAs from an MCF7 cell cDNA library and four from a ZR 75 cell cDNA library. Only two RNAs were isolated from both cDNA libraries even though all the regulated sequences are present in both cell lines. The detection of a regulated RNA by differential hybridization probably depends upon a combination of its abundance and degree of induction, and this may explain why most RNAs were isolated from only one library. For instance the pNR-1 and pNR-17 RNAs are considerably more abundant and also more induced in MCF7 cells than in ZR 75 cells while the pNR-7, pNR-13, pNR-20, pNR-21, and pNR-22 are
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FIG. 6. Effects of different steroids on the induction of the pNR-1, pNR-2, pNR-17, and pNR-25 RNAs. MCF7 cells were withdrawn and then cultured in the presence of dexamethasone, 10^{-9}, and 10^{-7} M, (Dex); estradiol, 10^{-8} M, (E); 5α-dihydrotestosterone, 10^{-8} M, (DHT); progesterone, 10^{-7} M, (Prog). Total RNA was prepared and analyzed as described under "Materials and Methods." The sizes of the RNAs are shown on the right in nucleotides.

of similar abundance in the two cell lines but are more induced in MCF7 cells (Table IV). The pNR-100 and pNR-101 RNAs, that were only isolated from the ZR 75 cDNA library, and the pNR-23 RNA, that was isolated from the MCF7 cDNA library, are abundant and regulated in both cell lines, and we currently have no explanation as to why they were only isolated from one cell line.

The four sequences isolated from the ZR 75 library are relatively abundant, and this suggests that the differential screening of the MCF7 library was more sensitive than the screening of the ZR 75 library. This may have resulted from the use of nylon rather than nitrocellulose membranes to screen the ZR 75 library.

Our results show that estrogen-responsive breast cancer cell lines have different patterns of expression and regulation of estrogen-responsive genes and suggest that new estrogen-regulated RNAs may be discovered by screening cDNA libraries prepared from other cell lines. Our results also emphasize the importance of screening large numbers of recombinants to isolate unabundant RNAs. This is, first, to ensure that recombinants containing a rare RNA are contained within the library and, second, that they are detected because their hybridization signals are more likely to be obscured by background. The second problem could be minimized by ensuring that recombinants contain long cDNA inserts.

Identification of Estrogen-regulated Sequences—Two other groups have screened MCF7 cDNA libraries by differential hybridization for estrogen-regulated sequences (10, 11). Both isolated a single, identical RNA, called pS2 by Masiakowski et al. (10). It is probable that only the pS2 RNA was isolated because small libraries (4000 (10) and 1120 recombinants (14)) were screened.

We have now confirmed by sequencing, that the pS2 RNA is identical to the pNR-2/105 RNA isolated by ourselves (14, 22). This RNA is readily identified by virtue of its abundance and large induction by estrogen. It codes for a small cysteine-rich protein with structural features reminiscent of IGF-1, prompting the speculation that it may exert an autocrine growth factor activity. It has only been detected in estrogen-responsive breast cancer cell lines in which it can be induced up to 100-fold by estrogens.

The pNR-100 mRNA, isolated from ZR 75 cells, has recently been sequenced and shown to encode the aspartyl lysosomal protease cathepsin D (9). This RNA may code for the estrogen-induced 46-kDa-secreted protein that we have identified previously (22) as this protein has recently been shown to have an acid carboxylase-like activity (23). Although the 46-kDa protein has the predicted molecular mass of cathepsin D, it has been reported to have a different cellular distribution and amino acid composition (9). The pNR-100 RNA is expressed in all the cell lines tested but is only regulated by estrogen in the estrogen-responsive breast cancer cell lines. It is tempting to speculate that the secretion of this normally lysosomal protease may enhance tumor growth and metastasis by tissue remodeling or destruction of basement membrane and components of the extracellular matrix. This process may be accentuated by estrogens in estrogen receptor positive breast tumors.

Although the pNR-1 RNA has been partially sequenced, we have not detected any identical or related sequences in the nucleic acid databases. The pNR-1 RNA is induced up to 20-fold in MCF7 cells and is only detected in estrogen-responsive breast cancer cell lines. Interestingly, this RNA is also induced by the antiestrogen, tamoxifen (24), and should therefore prove to be useful for studying the molecular mechanisms of antiestrogen action.

Less is known about the other 10 estrogen-regulated RNAs. With the exception of pNR-17 and pNR-25, the others are expressed in all the cell lines examined. It is possible that some of them code for known estrogen-inducible proteins, of which the progesterone receptor is the best characterized in human breast cancer cells. As there are at least five different progesterone receptor RNA sequences of different molecular weight in MCF7 and T47D cells, and as it is more abundant in T47D cells (25), it is unlikely that any of the estrogen-regulated RNAs described here code for the progesterone receptor. Other RNAs have also been reported to be increased by estradiol in human breast cancer cells (26, 27) but their relatively small induction by estradiol and molecular weights preclude them corresponding to the RNAs that we have isolated.

Prognostic and Predictive Value—Endocrine therapy has become widely used in the treatment of advanced breast cancer and increasingly as adjuvant therapy for primary breast cancer. It is well established that the presence of the estrogen receptor protein does not necessarily predict responsiveness to endocrine therapy. The measurement of estrogen-inducible proteases in breast tumors might indicate that they contain a functioning estrogen receptor system. It has therefore been suggested that measurement of both estrogen and progesterone receptors should improve prediction of response to endocrine therapy. Unfortunately, a significant proportion of patients whose tumors contain detectable levels of the two receptors still fail to respond to hormonal therapy.

Three of the estrogen-regulated RNAs that we have iden-

1 F. E. B. May and B. R. Westley, unpublished data.
tified (pNR-1, pNR-2, and pNR-25) were only detected in the estrogen-responsive breast cancer cell lines (Table IV). The proteins encoded by these RNAs are therefore the most likely of the other regulated RNAs in all the cell lines. This may not, however, accurately reflect the situation in vivo. The levels of all the RNAs must therefore be measured in breast tumor samples and their predictive value evaluated by correlating the RNA levels with the response of the patients to endocrine therapy.

In addition to predicting responsiveness to endocrine therapy, the presence of estrogen and progesterone receptors predicts the disease-free interval and survival of breast cancer patients. It is possible that the estrogen-regulated sequences that we have isolated will also be of prognostic value in breast cancer.

Mechanisms of Estrogen Action—The availability of cloned probes for a number of estrogen-regulated RNAs should enhance the usefulness of estrogen-responsive breast cancer cell lines for studying the mechanism of estrogen action.

The current working model for steroid hormone action suggests that the steroid binds with high affinity to a receptor protein. Binding is thought to induce a conformational change in the receptor that allows the steroid-receptor complex to interact with a cis-acting enhancer element in the chromatin, close to the regulated gene. This interaction somehow renders the promoter more effective, thereby increasing transcription of the responsive gene.

In this study we observed a large variation in both the constitutive level and degree of induction of the estrogen-regulated RNAs. Interpreted in the light of the above model, this suggests that the strengths of the promoters for the different estrogen-regulated genes and the effects of the hormone-inducible enhancers vary considerably. Thus, for example the pNR-2 and pNR-25 genes may have weak promoters in the absence of estrogen and enhancers that are highly estrogen responsive, whereas the pNR-101 gene may have a relatively strong promoter but weakly inducible enhancer.

The observation that both the constitutive levels and maximal induction of the RNAs varied enormously in the three estrogen-responsive cell lines tested, suggests that other factors are able to modulate the effects of both promoters and enhancers. The patterns observed were specific for each RNA; for instance the pNR-7 RNA was regulated most in the T47D cell line, whereas the pNR-20 RNA was less induced in this cell line.

It has recently been claimed that the presence of the estrogen receptor alone makes a cell estrogen responsive and that varying levels of these factors could explain the different regulation of the estrogen-regulated genes observed in the different cell lines. The absence of such factors could also account for the nonresponsiveness of some estrogen receptor positive breast tumors.

The estrogen-regulated genes have different sensitivities to estradiol. The model for estrogen action can be expanded to account for the differing dose dependence of the responsive genes to estradiol by proposing that the enhancer elements of the regulated genes have different affinities for the estradiol-estrogen receptor complex. Thus at a given concentration of estradiol, a certain fraction of the receptors will be bound to steroid, and the proportion of the enhancer elements of any gene occupied by the complex would depend on the affinity of the enhancer for the estradiol-estrogen receptor complex. Such a model would predict that the pNR-1 enhancer has a higher affinity for the estradiol-estrogen receptor complex than the pNR-25 enhancer.

The induction of the estrogen-regulated RNAs was generally specific for estrogens, however, there were some exceptions. For instance 5α-dihydrotestosterone and progesterone induced the pNR-1, pNR-2, and pNR-25 RNA levels while dexamethasone increased pNR-25 RNA levels but decreased pNR-1 and pNR-17 RNA levels. 5α-Dihydrotestosterone is known to interact with the estrogen receptor. It is therefore possible that the 5α-dihydrotestosterone-estrogen receptor complex could interact with the estradiol-inducible enhancer element in these genes but be less capable of rendering the promoter effective, perhaps as a result of a lower affinity of the receptor complex for the enhancer element. Alternatively 5α-dihydrotestosterone could act through the androgen receptor.

This complex might interact with the estradiol-inducible enhancer element or there might be another cis-acting element that interacts specifically with the 5α-dihydrotestosterone-androgen receptor complexes.

In this context it is interesting to note that the mouse mammary tumor virus promoter is induced by progestins (30) and androgens (31) as well as by glucocorticoids. In all three cases, the induction is mediated by the appropriate receptor. It is thought that the glucocorticoid and progesterin receptor complexes interact with the same enhancer element (32) and that the hormone regulatory element of the mouse mammary tumor virus may be primarily a progesterone responsive element (30). It is clear, however, that the controls of the estrogen-regulated RNAs differ both from each other and from those of the mouse mammary tumor virus RNA. Analysis of the regulatory sequences in the corresponding genes should provide an insight into the molecular basis for the differences.

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REFERENCES

1. Horwitz, K. B., Koseki, Y., and McGuire, W. L. (1978) Endocrinology 103, 1742-1751
2. Soule, H. D., Vasquez, J., Lang, A., Albert, S., and Brennan, M. A. (1970) J. Natl. Cancer Inst. 51, 1409-1416
3. Horwitz, K. B., Zava, D. T., Thilagar, A. K., Jensen, E. M., and McGuire, W. L. (1978) Cancer Res. 38, 2434-2437
4. Reddel, R. R., Murphy, L. C., Hall, R. E., and Sutherland, R. L. (1986) Cancer Res. 45, 1525-1531
5. Lippman, M. E., and Bolan, G. (1975) Nature 256, 592-593
6. Reddel, R. R., and Sutherland, R. L. (1984) Eur. J. Cancer Clin. Oncol. 20, 1419-1424
7. Darbre, P. D., Curtis, S., and King, R. J. B. (1984) Cancer Res. 44, 2790-2793
8. May, F. E. B., and Westley, B. R. (1986) Cancer Res. 46, 6034-6040
9. Westley, B. R., and May, F. E. B. (1987) Nucleic Acids Res. 15, 3773-3786
10. Masiakowski, P., Breathnach, R., Bloch, J., Gannon, F., Krust, A., and Chambon, P. (1982) Nucleic Acids Res. 10, 7985-7993
11. Prud'homme, J.-F., Friedlansky, P., Le Cunff, M., A., and Chambon, P. (1982) Nucleic Acids Res. 10, 7895-7903
12. Keydar, I., Chen, L., Karby, S., Weiss, F. R., Delarea, J., Radu, M., Chaitcik, S., and Brenner, H. J. (1979) Eur. J. Cancer Clin. Oncol. 15, 11-21
13. Engel, L. W., Young, N. A., Tralka, T. S., Lippman, M. E., O'Brien, S. J., and Joyce, M. J. (1978) Cancer Res. 38, 3352-3364
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14. Cailleau, R., Young, R., Olive, M., and Reeves, W. (1974) J. Natl. Cancer Inst. 53, 661–674
15. Lasfargues, E. Y., and Ozello, L. (1958) J. Natl. Cancer Inst. 21, 1131–1147
16. Polanowski, F. P., Gaffney, E. V. and Burke, R. E. (1976) In Vitro 12, 328–341
17. Gey, G. O., Coffman, W. D., and Kubicek, M. T. (1952) Cancer Res. 12, 264–265
18. Fabricant, R. N., Delarco, J. E., and Todaro, G. J. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 565–569
19. Westley, B. R., and May, B. R. (1984) Gese (Arnst.) 28, 221–227
20. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, D., and Green, G. R. (1984) Nucleic Acid Res. 13, 7035–7056
21. Jakowlew, S. B., Brestnach, R., Jeltsch, J-M., Maniakowski, P., and Chambon, P. (1984) Nucleic Acids Res. 12, 2961–2976
22. Westley, B., and Rochefort, H. (1980) Cell 20, 353–362
23. Morisset, M., Capony, F., and Rochefort, H. (1986) Biochem. Biophys. Res. Commun. 138, 102–109
24. May, F. E. B., and Westley, B. R. (1987) J. Biol. Chem. 262, 15894–15898
25. Miara, M., Atger, M., d’Auriol, L., Loosefelt, H., Meriel, C., Fridlansky, F., Guiochon-Mantel, A., Galibert, F., and Milgrom, E. (1987) Biochem. Biophys. Res. Commun. 143, 740–746
26. Lippman, M. E., Dickson, R. B., Gelmann, E. P., Rosen, N., Knabbe, C., Estes, S., Bronzert, D., Huff, K., and Kasid, A. (1987) J. Cell Biochem. 35, 1–16
27. Edwards, D. P., Adams, D. J., Savage, N., and McGuire, W. L. (1980) Biochem. Biophys. Res. Commun. 93, 804–812
28. Druge, P. M., Klein-Hitpass, L., Green, S. S. G., Chambon, P., and Ryffel, G. U. (1986) Nucleic Acids Res. 14, 9529–9537
29. Cordingly, M. G., Tate Riegel, A., and Hager, C. L. (1987) Cell 48, 261–270
30. Cato, A. C. B., Miksicek, R., Schütz, G., Arnemann, J., and Beato, M. (1986) EMBO J. 5, 2237–2240
31. Darbre, P., Page, M., and King, R. J. B. (1986) Mol. Cell. Biol. 6, 2647–2654
32. Ahe, D. v. d., Janich, S., Scheidereit, C., Renkawatz, R., Schütz, G., and Beato, M. (1986) Nature 313, 706–709