Tissue-specific and Hormonally Controlled Alternative Promoters Regulate Aromatase Cytochrome P450 Gene Expression in Human Adipose Tissue*

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Estrogen biosynthesis is catalyzed by a microsomal enzyme, aromatase cytochrome P450 (P450arom; the product of the CYP19 gene). The human CYP19 gene comprises nine coding exons, II-X. Additionally, tissue-specific expression is determined by the use of tissue-specific promoters, which give rise to P450arom transcripts with unique 5'-noncoding sequences. In placenta, P450arom transcripts contain one of two 5'-untranslated exons, I.1 or I.2, while ovarian transcripts instead contain sequence consistent with the use of a promoter, PII, which is proximal to the start of translation. To characterize transcripts present in adipose tissue and adipose stromal cells (ASC) in culture, cDNA libraries were constructed by the RACE (rapid amplification of cDNA ends) procedure. Four P450arom transcripts with unique 5' termini were identified, leading to the characterization of two unique 5'-untranslated exons of the CYP19 gene, L.3 and L.4. Whereas L.3-specific sequence is expressed in adipose tissue as well as in ACS maintained under all culture conditions, L.4-specific sequence is apparently present only in breast adipose tissue, and ACS stimulated with glucocorticoids. On the other hand, PII-specific sequence is present only in cells stimulated with cAMP analogues and is absent from cells stimulated with glucocorticoids. We conclude that CYP19 gene expression in human adipose tissue likely utilizes two novel promoters and, furthermore, that alternative promoter usage in cultured ASC is a function of the hormonal environment in which the cells are maintained.

The biochemical and physiological actions of estrogens include morphic anatomical, functional, and behavioral development of males and females that is essential for reproduction. Because of the importance of estrogen biosynthesis, understanding its regulation is essential to gain insights into the physiological and pathophysiological actions of estrogens. The biosynthesis of estrogens from androgens is catalyzed by an enzyme complex of the endoplasmic reticulum termed aromatase. The aromatase enzyme complex is composed of two polypeptides, NADPH-cytochrome P450 reductase, a ubiquitous flavoprotein of the endoplasmic reticulum, and aromatase cytochrome P450 (P450arom); the product of the CYP19 gene, a unique form of cytochrome P450, which appears to be present exclusively in estrogen-producing cells (1–5). The reaction involves three hydroxylation steps and requires NADPH-cytochrome P450 reductase as well as reduced equivalents to the P450arom, which binds the C19 substrate and catalyzes insertion of oxygen into the molecule, resulting in formation of the C18 estrogen (6–10).

In most species throughout the vertebrate phylum, estrogen biosynthesis is limited to the gonads and brain. In the human and other primates, however, estrogen production has a wider tissue distribution, which includes adipose tissue (11) and placenta (12). Studies showing an increased fractional conversion of circulating plasma androstenedione to estrone as a function of increased obesity as well as increased age implicate adipose tissue as a significant site of estrogen biosynthesis (13, 14) and as the major source of estrogens in postmenopausal woman and in elderly men. Unlike the ovary, estrogen production in adipose is not cyclic but continuous. While the physiological role of extragonadal estrogen production is not clear, there appears to be a relationship between estrogen biosynthesis in adipose and several disease states such as endometrial cancer, breast cancer, and osteoporosis, as well as chronic amenorrhea of obese women and gynecomastia in obese men. Clearly, understanding the regulation of estrogen biosynthesis in adipose tissue will provide insights into the role of estrogens in these conditions.

Both cell types found in adipose tissue, namely adipocytes and adipose stromal cells, express aromatase, although much higher aromatase activity and expression of CYP19 transcripts have been observed in stromal cells (15, 16). Adipose stromal cells in monolayer culture have been utilized as a model system to examine factors that regulate aromatase in vitro and to study CYP19 gene expression. A variety of factors have been shown to positively affect aromatase activity and P450arom expression in adipose stromal cells, including glucocorticoids as well as cAMP analogs and phorbol esters. The stimulatory effects of dexamethasone require the presence of fetal calf serum (FCS) (17) in the medium while the increases induced by dibutyryl cAMP (Bt2cAMP) or Bt2cAMP + phorbol diacetate (PDA) are manifest only in the absence of FCS (18). A variety of growth factors, including epidermal growth factor, fibroblast growth factor, platelet-derived growth factor, transforming...
growth factors α and β, and tumor necrosis factor, have been shown to mimic the inhibitory action of FCS on Bt2cAMP- or Bt2cAMP + PDA-dependent increases in CYP19 expression (18-19). Aromatase regulation by these agents is tissue-specific since in the ovary, glucocorticoids have little or no effect to stimulate aromatase activity and phorbol esters inhibit rather than potentiate the Bt2cAMP-induced stimulation of aromatase activity (20).

Isolation and characterization of the human CYP19 gene as well as the characterization of tissue-specific alternative promoters has led to the identification of one mechanism to explain the complex and tissue-specific regulation of P450 arom expression. The human CYP19 gene spans at least 75 kb and is comprised of nine coding exons, exons II-X (21-23). Additionally, two noncoding exons, exon I.1 and I.2, have been described previously (21). In placenta, the majority of CYP19 transcripts contain exon I.1 in the 5'-untranslated region (21, 24) and a minor population of transcripts contain exon I.2 (25). Genomic sequences identified upstream of exon I.1 have been shown to regulate CYP19 gene transcription in placental-derived choriocarcinoma cells (23, 26). Tissue-specific regulation of CYP19 expression by alternative promoters was first demonstrated in adipose stromal cells in culture. CYP19 transcripts in these cells do not contain either exon I.1 or I.2 sequences; thus promoters upstream of these exons most probably do not serve to regulate P450 arom expression in these cells. By primer extension and S1 nuclease analysis using probes spanning the first coding exon, adipose stromal cells maintained in the presence of Bt2cAMP+PDA have been shown to contain transcripts of which some 50% utilize a start site of transcription 26 bp downstream of a TATA-like sequence within the putative promoter-like sequence termed promoter II (PII), which is proximal to the start of translation (27). Additionally, the S1 nuclease protection assay was suggestive that at least 50% of adipose CYP19 transcripts may have an alternative transcriptional start site upstream of an exon that is not contiguous with exon II. The results of PCR experiments suggested that this exon(s) could not be either exon I.1 or I.2 and raised the possibility of another untranslated exon in the CYP19 gene, the transcription product of which is spliced onto exon II during processing of adipose stromal cell transcripts (27). CYP19 transcripts in the ovary also do not contain the placenta-specific exons I.1 and I.2, but the majority of transcripts have a start site of transcription 26 bp downstream of the TATA-like sequence within the putative promoter PII (24). Thus alternative promoters regulate CYP19 transcription, giving rise to P450 arom mRNAs with tissue-specific 5’-untranslated sequences. These untranslated exons are spliced onto a common 3’-splice site upstream of the translational start site; thus the coding sequence is the same in all tissues.

The observation that additional transcriptional start sites might be utilized in adipose stromal cells led us to evaluate CYP19 transcripts in these cells as well as in adipose tissue for the presence of novel 5’-noncoding sequences. Through construction of cDNA libraries from adipose tissue and adipose stromal cells, exon-specific Northern analysis, and screening of genomic libraries, this work has led to a number of interesting and novel developments including the identification of two unique 5’-untranslated exons of human CYP19, as well as the observation that alternative promoter usage in adipose stromal cells in culture is a function of the hormonal environment in which the cells are maintained. A preliminary account of this work has been presented elsewhere (28).
RESULTS

Characterization of cDNA Libraries Prepared from Adipose Tissue and Adipose Stromal Cells—A putative promoter region, PII, 110 bp upstream of the first coding exon, namely exon II, was previously identified as a possible regulatory region for CYP19 transcripts in adipose stromal cells in culture treated with Bt2cAMP and PDA (27), and in corpus luteum tissue of the ovary (28). The identification of this putative promoter region was based solely on the identification of a transcriptional start site just downstream of a TATA-like sequence by S1 nuclease protection and primer extension analysis of adipose stromal cell and corpus luteum mRNA. To determine whether promoter II was capable of mediating transcriptional regulation in human adipose tissue, 935 bp of PII and upstream flanking sequence was ligated to a CAT reporter gene and transfected into human adipose stromal cells. Transfection of the -935AROM-CAT reporter construct into adipose stromal cells resulted in very low levels of CAT activity, which were not appreciably induced by cAMP + PDA, whereas an RSVCAT construct containing the Rous sarcoma virus promoter linked to the CAT gene was capable of initiating transcription in these cells (data not shown). This observation suggested several possibilities: 1) sequences further upstream than -935 are required for promoter II activity; 2) sequences downstream of the first coding exon are required for promoter II activity; or 3) promoter II is not the major promoter regulating CYP19 expression in human adipose.

Based on the evidence of the previously published S1 nuclease protection analysis (27) suggesting different transcriptional start sites in addition to PII-specific CYP19 transcripts, as well as the initial transfection studies employing PII-specific reporter constructs, it appeared likely that adipose stromal cells in culture contain CYP19 transcripts with alternative 5′ ends that are regulated by alternative promoters. To characterize the genomic regulatory regions upstream of these exons, it was important to identify unique sequences at the 5′ ends of CYP19 transcripts in adipose tissue and in cultured stromal cells. Once these exons were identified, their sequences could be used as probes to determine their location relative to the rest of the CYP19 gene and thus to identify novel regulatory elements upstream of these untranslated exons.

Since the possibility existed that adipose tissue transcripts might contain more than one 5′-untranslated sequence, it was advantageous to construct the cDNA libraries so that many CYP19 cDNA clones could be readily isolated. Obtaining full-length cDNA clones of low abundance RNAs has proven to be difficult and laborious using traditional methods of cDNA cloning. However, utilizing a relatively new method of cDNA synthesis by PCR, a large number of cDNA clones can be identified rapidly. The method termed RACE (rapid amplification of cDNA ends) (30, 31) allows for synthesis of primer-extended cDNA libraries using the DNA polymerase chain reaction technique to amplify copies of the sequence between a single region in the transcript and the 3′ or 5′ end. The minimum information required for this amplification is a single short stretch of sequence within the mRNA to be cloned. However, since the RACE procedure utilizes the PCR method, which can give rise to differential amplification of particular transcripts, no firm conclusions can be reached as to the relative abundance of any newly identified sequence based solely on RACE identification.

RACE cDNA libraries were constructed from adipose tissue, as well as adipose stromal cells that were either left untreated or treated with dexamethasone or Bt2cAMP + PDA. By making libraries from cells in culture under the various hormonal conditions, one can then establish if there are new 5′ termini and thus determine if alternative promoters are employed in response to changes in the hormonal environment of the cells.

A number of cDNA clones from each library were sequenced in order to estimate the distribution of the alternative 5′ sequences. As indicated in Table I, in addition to the promoter I1-specific sequence, four new 5′ sequences were identified (Fig. 1). These new sequences are referred to as I3 and I4 in the order that they were discovered, and I3-truncate and I4L2. All of the new sequences were spliced onto exon II at the same 3′-splice junction as exons I.1 and I.2, upstream of the start of translation, and thus would be expected to encode the same protein. The distribution of the various 5′ sequences seemed to be influenced by the hormonal treatment of the cells (Table I). In adipose tissue, I4.1, I3, and I3-truncate sequences are present. Three cDNA libraries were made from adipose tissue; two were from breast adipose of two different patients, and the other from thigh/calves. No I4-containing CYP19 transcripts were identified in the library made from thigh/calf tissue, and no I3 truncate-containing CYP19 transcripts were identified in the libraries made from breast adipose tissue. The difference in distribution of 5′ ends in the three adipose tissue libraries could be due to patient to patient variation, or else it could be a function of tissue localization. In the stromal cells in culture, the choice of 5′ termini appears to be dependent on the hormonal environment of the cells. Most interestingly, in cells expressing PII-specific sequences, namely cells treated with cAMP + phorbol esters in the absence of serum, no I4-specific sequences are detected. Conversely, in cells expressing I4-specific sequences such as dexamethasone-treated cells in the presence of serum, no PII-specific sequences are observed. P450arom transcripts containing exon I.3 are present in adipose tissue as well as in cells in culture under all conditions.

Sequence analysis of the cDNAs revealed that the I.3 and I3-truncate cDNAs were identical at their 5′ ends (Fig. 1). Based on the longest cDNA isolated, the I.3-specific sequence is 205 bp and the I3-truncate-specific sequence contains 99 bp of the most 5′ sequence of exon I.3. The possibility exists that both transcripts are driven by a common promoter. Based on the abundance of I.3 cDNA clones versus I3-truncate clones, it is likely that P450arom mRNA species containing I.3 sequences are present in higher levels than those containing I3-truncate.
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Fig. 1. Sequence of the five P450arom cDNAs identified in adipose tissue and cells by the RACE procedure. The sequences indicated represent the longest cDNA clone identified containing each particular 5' sequence. The four new 5' sequences and PII-specific sequences are underlined. All four of the new 5' sequences are spliced to the 5' end of exon II at the same splice junction.

Fig. 2. Diagram of the five cDNAs isolated by the RACE procedure. The diagram shows in schematic form the regions of identity between exons I.3 and I.3-truncate and between exons I.4 and I.4/12.

Fig. 3. Northern analysis of adipose tissue and adipose stromal cell poly(A)+ RNA. RNA was prepared from a pool of breast and abdominal adipose tissue from several subjects. The relative use of PII, I.3-, and I.4-containing sequences was determined under various hormonal conditions. As indicated on the left, the RNA blot was repeatedly probed using 65-75-bp probes specific to an exon in the coding region (exon I), as well as PII, I.3, and I.4 sequences. The probes were amplified and labeled by asymmetric PCR. Lane 1, 40 μg of poly(A)+ RNA from whole adipose tissue; lane 2, 40 μg of poly(A)+ RNA from adipose stromal cells in culture + FCS; lane 3, 20 μg of poly(A)+ RNA from adipose stromal cells in culture + FCS + 250 nM dexamethasone; lane 4, 20 μg of poly(A)+ RNA from adipose stromal cells in culture + FCS + 0.5 mM Bt2cAMP; lane 5, 20 μg of poly(A)+ RNA from adipose stromal cells in culture + FCS + 0.5 mM Bt2cAMP + 100 nM PDA; lane 6, 5 μg of placent poly(A)+ RNA; lane 7, genomic DNA positive control specific to PII sequences and exon I.3; lane 8, DNA positive control for the exon I.4 probe; lane 9, blank; lane 10, DNA positive control for the exon I.3 probe; lane 11, DNA positive control for the exon I.4 probe.

The various 5' ends as probes (Figs. 3 and 4). Poly(A)+ RNA from adipose tissue, as well as adipose stromal cells cultured in control medium in the absence of FCS, cells treated with dexamethasone plus FCS, or else with Bt2cAMP or Bt2cAMP + PDA in the absence of FCS, were probed with 65-75-bp fragments specific for each 5' end. The probes were labeled to roughly equal specific activity, and equivalent amounts of radiolabeled cDNA were used in the hybridizations.

Consistent with our findings regarding the RACE libraries, promoter II-specific transcripts were detectable in 20 μg of poly(A)+ RNA from adipose stromal cells in culture treated with Bt2cAMP or Bt2cAMP + PDA, but not in the same amount of poly(A)+ RNA from cells treated with dexamethasone (Fig. 3). The RNA blot in Fig. 4, which contained 40 μg of poly(A)+ RNA/lane, was also probed with a PII-specific fragment, and
This is the first conclusive evidence of differential expression of RNA was prepared from breast adipose tissue from different donors amethasone-treated adipose stromal cells (data not shown). again no hybridization was detected in poly(A)+ RNA from dexamethasone-treated adipose stromal cells cultured + serum; lane 3, 40 μg of poly(A)+ RNA from cultured adipose stromal cells in the presence of serum and 250 ng dexamethasone. Exposure times were 4 days in the case of exon IX and I.4 probes and 7 days in the case of the I.5 probe.

Again no hybridization was detected in poly(A)+ RNA from dexamethasone-treated adipose stromal cells (data not shown). This is the first conclusive evidence of differential expression of 5' termini under different culture conditions. While a single cDNA clone amplified from control cells maintained in the absence of FCS contained PII-specific sequence, no hybridization to PII-specific probes was detected by Northern analysis, suggesting that only a very few P450arom transcripts in untreated cells contain this sequence. As can be seen in Fig. 3, Northern analysis using a probe specific for both I.3 and I.3-truncate sequences revealed hybridization to RNA from control cells maintained in the absence of FCS, from cells treated with Bt2cAMP, or Bt2cAMP + PDA, but not from glucocorticoid-treated cells. This was unexpected since I.3-containing cDNA clones were identified in libraries made from RNA isolated from dexamethasone-treated adipose stromal cells (Table I). However, subsequent Northern analysis of 40 μg of poly(A)+ RNA from dexamethasone-treated adipose stromal cells, as well as untreated cells in the absence and presence of FCS, revealed hybridization to an exon I.3-specific probe, although for dexamethasone-treated samples the level of I.4-specific transcripts appeared to be several-fold greater than the level of I.3-specific transcripts (Fig. 4). The fact that the abundance of I.3-containing transcripts in dexamethasone-treated cells is low compared to the abundance of I.4-containing transcripts may explain the lack of I.3-containing transcripts detectable in Fig. 3, where only 20 μg of poly(A)+ RNA was used. Alternatively, since the RNA used in the experiment shown in Fig. 3 was prepared from a pool of breast and abdominal adipose tissue, it is possible that the RNA used in Fig. 4 was from breast adipose tissue from two different donors, there may be a region-specific difference in the relative expression of these transcripts. A I.4-specific probe hybridized only to RNA from dexamethasone-treated cells in the presence of FCS (Figs. 3 and 4). This result is consistent with the absence of P450arom cDNAs containing exon I.4 in libraries from Bt2cAMP- or Bt2cAMP+PDA-treated cells. Additionally, no hybridization of the I.4-specific probe was detected in transcripts from untreated cells either in the absence or presence of FCS (Fig. 4). While dexamethasone-dependent increases in aromatase expression occur only in the presence of FCS, the lack of I.4 transcripts in untreated cells maintained in the presence of FCS suggests that glucocorticoids have a direct action to regulate expression of I.4-containing transcripts, together with growth factor(s) present in the serum. On the other hand, a probe specific for exon I.2 failed to hybridize under any conditions, suggesting that I.4/I.2 containing transcripts are present in very low abundance (data not shown). This observation is consistent with the low number of cDNA clones that contain this particular 5' end. These results obtained employing exon-specific Northern analysis are summarized in Table II.

Northern analysis showed that 40 μg of poly(A)+ RNA from adipose tissue failed to hybridize to any CYP19 probe (Fig. 3, lane 1), while a probe specific to the adipose-specific glucocorticoid transporter, Glut 4 (34, 35), showed readily detectable hybridization (data not shown). Thus although the RNA is intact, CYP19 transcripts in whole adipose tissue are present at levels too low to be detected by Northern analysis. As an additional means to verify the presence of exons I.4 and I.3 in adipose tissue, RT-PCR was used to amplify exons I.3 and I.4 separately and I.3 or I.4 together with exon II (Fig. 5). Total RNA from breast adipose tissue was used as a template for first strand cDNA synthesis. Exon II, the first coding exon, was amplified alone as a positive control. The untranslated exons I.3 and I.4 were amplified together with exon II and so should represent only spliced products. As shown in Fig. 5 (lanes 4 and 6), both exons I.3 and I.4 were readily amplified from adipose tissue in agreement with the observed RACE cDNAs. In lane 6 three bands can be observed. The largest and faintest band at 488 bp, represents unspliced RNA products containing exon I.3, a 100-bp intron, and exon II. The second band, at 388 bp, represents exons I.3 and II together. The smaller band, at 282 bp, represents the I.3-truncate sequence spliced onto exon II. This experiment is in agreement with the results obtained using the RACE cDNA clones, namely that in whole breast adipose tissue, CYP19 transcripts have alternative 5' ends, which include exons I.3 and I.4. However, since both analyses were based on PCR, no conclusions can be made with respect to the relative abundance of these transcripts.

Genomic Localization of New CYP19 Untranslated Exons —At this point it was evident that alternative CYP19-specific 5' termini are expressed in a tissue-specific fashion and that, additionally, these alternative 5' sequences are expressed in adipose stromal cells cultured under different conditions. Since promoter sequences are generally located just 5' of the start site of transcription, expression of these alternative 5' ends may be regulated by alternative promoters. Having identified these new 5' sequences, it was then necessary to determine their location in the CYP19 gene, in order to identify the putative promoter sequences regulating expression of these alternative CYP19 transcripts. Upon comparison of exon I.3 to sequences upstream of exon II, exon I.3 was found to be located just upstream of exon II

| Table II | Summary of major 5' termini in adipose cells and tissues |
| Tissue cells | Major 5' terminus |
| Adipose tissue | I.4, I.3 |
| Adipose stromal cells in culture | |
| Control – serum | I.3 |
| Control + serum | I.3 |
| Dex + serum | I.4, I.3 |
| cAMP – serum | PII/I.3 |
| cAMP + PDA – serum | PII/I.3 |
| Ovary | PII |
| Placenta | I.1 |
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Since the genomic clones containing exons 1.1 and 1.2 did not overlap, a gap remained in the CYP19 gene in the region between exon 1.1 and exon 1.2. In consideration of the possibility that exon 1.4 was located in this region, a human genomic library was screened in hopes of isolating the remainder of the gene. An EMBL-3 SP6/T7 human genomic library was screened using a PCR-labeled 65-bp exon 1.4-specific fragment as a probe. A single, 16-kb positive clone was obtained after screening 450,000 plaque-forming units (Fig. 7). The 16-kb insert was cut out of λ using SacI to release 1.3-, 8.5-, 1.5-, and 4.5-kb fragments. Southern analysis using the exon 1.4 probe indicated the presence of exon 1.4 in the 4.5-kb SacI genomic fragment.

In an attempt to determine if the 1.4-containing genomic fragment overlapped with genomic clones containing either exons 1.1 or 1.2, or both, Southern analysis was performed using random-primer labeled fragments corresponding to the 3’ end of the exon 1.1-containing genomic fragment or the 5’ end of the 1.2-containing genomic fragment. No hybridization was detected with the 1.2-containing fragment, indicative that the 1.4 clone did not overlap this region of the gene. However, the 1.1-containing genomic fragment did hybridize to the 1.4-containing genomic clone, verifying that these two clones overlapped. Through restriction mapping and Southern analysis, untranslated exon 1.4 was estimated to be located 20 kb downstream of exon 1.1. The size of the overall gene is therefore estimated to be at least 75 kb. Our current knowledge of the organization of the CYP19 gene upstream from exon II is summarized in Fig. 6, which additionally shows the various splicing possibilities that we have detected.

Characterization of Exon 1.4—The 4.5-kb SacI fragment containing exon 1.4 was subcloned and sequenced (Fig. 8). Upstream from the end of the longest sequence found in the cDNA clones there are no obvious TATA, CAAT, or GC-rich sequences, which might serve as possible promoter regions. At the same time, the size of I.4-containing transcripts, as indicated by Northern analysis (Figs. 3 and 4), is such that we would not expect the exonic sequence to extend much further upstream. Primer extension and S1 nuclease protection analysis will have to be undertaken to define the promoter sequences in this region of the gene.

DISCUSSION

Tissue-specific Expression of Human P450arom—Aromatase is expressed throughout the entire spectrum of the vertebrate phylum. In most species however, expression is confined to the gonads and the brain. In humans and some other higher primates, expression occurs additionally at other sites, in particular, placenta and adipose tissue. Based on the results of this and previous studies, we conclude that in the human, tissue-specific expression of the CYP19 gene is under the control of tissue-specific promoters. The resulting transcripts are generated by means of alternative splicing. Thus, expression in ovary appears to be under the control of a promoter, which is proximal to the start of translation (24), similar to the situation in rat (36) and chicken (37). This is likely, therefore, to be the primordial promoter regulating CYP19 expression. When in the course of evolution the human placenta developed the ability to synthesize estrogens, this promoter was not utilized. Instead, transcripts from placenta contain sequences that are at least 35 kb upstream from the start of translation, apparently under the regulation of a distal promoter I.1 (21). This helps to explain the differences in the regulation of aromatase expression in these two tissues. By contrast, transcripts in adipose contain two different 5’ termini, which we have named exon 1.3 and exon 1.4. Exon 1.3 is located just 100 bp upstream of exon II, transcripts containing exon 1.3 are formed as a result of a small

FIG. 6. Structure of the human CYP19 gene and alternative splicing patterns upstream of the translational start site. The four untranslated exons and first coding exon (exon II) are indicated. Promoters I.1 and II and putative promoters I.4, I.2, and I.3 are also indicated. The size of the genomic region shown spans a distance of at least 35 kb, but since the genomic clones containing exons I.1 and I.4, on the one hand, and exon I.2, on the other, have not been overlapped, the true distance is still unknown.

(Fig. 6). A 100-bp intron exists between exons I.3 and II such that promoter II and some of its upstream regulatory sequences are included as exonic sequences in exon I.3-containing P450arom transcripts. Based on the largest cDNA clone isolated containing exon I.3, exon I.3 appears to be approximately 205 bp. It should be noted that a second proximal TATAA sequence exists in the gene 20 bp upstream of the start of translation, apparently under the control of a distal promoter, which is proximal to the start of translation (24), similar to the situation in rat (36) and chicken (37). This is likely, therefore, to be the primordial promoter regulating CYP19 expression. When in the course of evolution the human placenta developed the ability to synthesize estrogens, this promoter was not utilized. Instead, transcripts from placenta contain sequences that are at least 35 kb upstream from the start of translation, apparently under the regulation of a distal promoter I.1 (21). This helps to explain the differences in the regulation of aromatase expression in these two tissues. By contrast, transcripts in adipose contain two different 5’ termini, which we have named exon 1.3 and exon 1.4. Exon 1.3 is located just 100 bp upstream of exon II, transcripts containing exon 1.3 are formed as a result of a small

FIG. 5. RT-PCR amplification of exons I.3 and I.4 from breast adipose tissue. The amplified products were separated on a 2% agarose gel, visualized using ethidium bromide staining (panel A), transferred to Zeta-Probe (Bio-Rad), and probed with an end-labeled oligonucleotide hybridizing to the middle of exon I.1 (panel B). Lane 1, 123-bp molecular weight ladder; lane 2, PCR amplification using exon II-specific primers; lane 3, PCR amplification using exon I.4-specific primers; lane 4, PCR amplification of exon I.4 together with exon II; lane 5, PCR amplification using exon I.3-specific primers; lane 6, PCR amplification of exon I.3 together with exon II; lane 7, 1-kb molecular weight ladder; lane 8, no RNA control. The expected sizes of the amplified products were: exon II, 183 bp; exon I.4 together with exon II, 248 bp; exon I.3 together with exon II, 389 bp.
splicing event and, in fact, contain some of the promoter II region as exonic sequence. Exon I.4 is located 20 kb downstream from exon I.1 and therefore is at least 15 kb upstream from the start of translation. However, since the clone bearing this sequence does not overlap those containing exons I.2 and II, the actual distance is not yet known.

Additionally, a number of other splicing events occur in various tissues giving rise to minor CYP19 transcripts. For example, in placenta, a small percentage of transcripts contain exon I.2, located 9 kb upstream of the start of translation (25). Additionally, in adipose tissue there is a truncated form of I.3, additional to exon 1.4 has recently been reported to be present in 5' termini of CYP19 transcripts in adipose stromal cells in culture (26). Exon 1.4 is located 20 kb downstream of exon 1.1 by 5.7 kb, but does not overlap the exon I.2-containing genomic fragment. Exon I.4 is located 20 kb downstream of exon I.1.

Hormonal Regulation of Promoter Selection in Adipose Stromal Cells in Culture—One of the most intriguing aspects of the present study is the observation that the distribution of 5'-terminal of CYP19 transcripts in adipose stromal cells in culture is a function of the culture conditions. In particular, promoter II-specific transcripts are present only in cells maintained in the presence of dibutyryl cyclic AMP plus phorbol ester or else dibutyryl cyclic AMP alone, in the absence of FCS. By contrast, transcripts containing exon I.4 are absent under these culture conditions. On the other hand, exon I.4-containing transcripts are present in cells maintained in the presence of dexamethasone plus FCS, whereas promoter II-specific transcripts are entirely absent under these conditions. Exon I.3-specific transcripts on the other hand, appear to be present under all culture conditions including those in which cells are maintained in the absence of stimulatory factors. Based on these findings, we propose that expression of transcripts containing exon I.4 is glucocorticoid-specific and, in addition, requires the presence of serum or growth factors. Thus, glucocorticoid stimulation of expression mediated by putative promoter I.4 would lead to the formation of transcripts containing exon I.4 in their 5' termini. By contrast, expression of promoter II-specific sequences appears to be cyclic AMP-mediated. This concept is consistent with the finding that, in the ovary, transcripts specific for promoter II are uniquely present (24), whereas those specific for exon I.4 are undetectable. CYP19 expression in human granulosa cells is known to be stimulated by cyclic AMP analogs but not by glucocorticoids. Similarly, a sequence apparently identical to exon I.4 has recently been reported to be present in 5' termini of CYP19 transcripts in human skin fibroblasts, cells in which aromatase activity is known to be induced by glucocorticoids (38).

An important question that then arises is whether or not enhancement of expression of transcripts from promoters II and I.4 by cyclic AMP and dexamethasone, respectively, is suf-
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