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Cathepsin D gene is controlled by a mixed promoter, and estrogens stimulate only TATA-dependent transcription in breast cancer cells

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ABSTRACT **The cathepsin D (cath-D) gene, coding for a ubiquitous lysosomal aspartyl protease, is overexpressed in aggressive human breast cancers, and its transcription is induced by estrogens in hormone-responsive breast cancer cells. We have determined the structure and function of the proximal 5′ upstream region of the human cath-D gene from MCF7 cells. We show that the promoter has a compound structure with features of both housekeeping genes (high G+C content and potential transcription factor Sp1 sites) and regulated genes (TATAA sequence). By RNase protection assay, we show that transcription is initiated at five major transcription sites (TSSI to -V) spanning 52 base pairs. In hormone-responsive breast cancer cells, estradiol increased by 6- to 10-fold the level of RNAs initiated at TSSI, which is located about 28 base pairs downstream from the TATA box. The specific regulation by estradiol of transcription starting at site I exclusively was confirmed by primer extension. Moreover, the same estradiol effect was observed in the ZR75-1 cell line and in MDA-MB231 estrogen-resistant breast cancer cells stably transfected with the estrogen receptor. Site-directed mutagenesis indicated that the TATA box is essential for initiation of cath-D gene transcription at TSSI. In breast cancer biopsy samples, high levels of TATA-dependent transcription were correlated with overexpression of cath-D mRNA. We conclude that cath-D behaves, depending on the conditions, as a housekeeping gene with multiple start sites or as a hormone-regulated gene that can be controlled from its TATA box.**

Steroid receptors increase the initiation of transcription of specific genes by interacting with the transcriptional machinery at the promoter level (1). Cathepsin D (cath-D), a lysosomal aspartyl protease, is induced by estrogens in human breast cancer cell lines (2) and is produced in excess in cancer cells both *in vitro* and *in vivo*, where its concentration in the primary tumor is correlated with increased risk of metastasis (for review see ref. 3).

In eukaryotes, gene expression is controlled by both proximal and distal elements, generally located in the 5′ upstream region of the gene (4). Many class II gene promoters contain a TATA box, which binds the transcription factor IID and defines the transcription initiation site; genes with these promoters have been called facultative or regulated genes. In contrast, promoters of housekeeping genes, such as those coding for lysosomal enzymes (5), lack a recognizable TATA box but contain multiple GC boxes acting as putative binding sites for the transcription factor Sp1 (6).

We have previously shown, in human breast cancer cell lines, that estrogens stimulate transcription of the cath-D gene by means of estrogen-responsive sequences located in the proximal region of the promoter (7). Here we show, using RNase protection and primer extension, that cath-D gene transcription in breast cancer cells is initiated at multiple sites by a mixed promoter having characteristics of the promoters of both housekeeping and hormonally regulated genes and that estradiol stimulates exclusively TATA-dependent transcription.

MATERIALS AND METHODS

Constructs and Probes. The antisense RNA probe used to map 5′ ends of endogenous cath-D mRNA was synthesized from plasmid pSS609, which contains the −194/+125 *Sma* I fragment of the cath-D gene inserted into the *Sma* I site of the plasmid pspt18. After linearization by *Eco*RI, this probe was transcribed in the presence of [^{32}P]UTP (400 Ci/mmol, Amersham; 1 Ci = 37 GBq) with T7 RNA polymerase according to the supplier's instructions (Sp6/T7 transcription kit from Boehringer). The human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control probe [804-base-pair (bp) *Xba* I/*Pst* I fragment of the GAPDH cDNA subcloned in the pspt19 vector] was synthesized with Sp6 RNA polymerase after digestion by *Hin*dIII.

CD3510 recombinant contains the −366/−13 *Xma* III fragment of the cath-D promoter inserted in the *Bam*HI/*Bgl* II sites of pBLCAT8+ (8). The pEH5 probe was generated by subcloning the *Hin*dIII/*Eco*RI fragment of CD3510 in the *Hin*dIII/*Eco*RI sites of pspt18. ^{32}P-radiolabeled antisense RNA was synthesized with T7 RNA polymerase after being cut with *Hin*dIII. Oligonucleotide-directed mutagenesis was used to create the TATA mutant derivative of CD3510 (TmutCD354) (*in vitro* mutagenesis kit from Amersham). The GCTATAAGC sequence was replaced by GCT*CT*G*C*GC by using a 31-mer synthetic oligonucleotide. The corresponding TmutpEH17 probe used in the RNase protection assay was generated as described for pEH5.

The probes for primer extensions included a 36-mer oligonucleotide (from positions +61 to +97 on the cath-D cDNA; see Fig. 1*D*) to map the 5′ ends of endogenous cath-D mRNA and a 32-mer oligonucleotide (from positions 1762 to 1793 in the coding sequence of the chloramphenicol acetyltransferase (CAT) gene; see Fig. 3*A*) to map transcription initiation sites from the transiently transfected CD3510 and TmutCD354 constructs.

Cell Culture and DNA Transfection. MCF7, ZR75-1, MDA-MB231, and BT20 cells were cultured in Dulbecco's modified Eagle's medium supplemented with fetal calf serum (GIBCO). To study the effect of estrogens, cells were switched to medium without phenol red containing charcoal-treated serum. After at least 5 days of withdrawal, cells were treated or not with 10 nM estradiol for 24 or 48 hr. DNA transfection was performed by calcium phosphate precipitation (9). One day after transfection of 20 μg of DNA, cells were washed and cultured for another day in medium sup-

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Abbreviations: cath-D, cathepsin D; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TSS, transcription start site; ER, estrogen receptor.

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plemented with 10% fetal calf serum in the presence of 10 nM estradiol. The MDA-MB231 HEGO transfected clones are described elsewhere (10).

RNA Preparation and Analysis. Total RNA was extracted by using the LiCl/urea method (11). The transcription initiation sites of cath-D gene were determined by protection from RNase digestion and primer extension (ref. 12, pp. 7.74–7.83). For RNase protection assay, the hybridization was performed, using 10 μg of RNA, overnight at 50°C for pSS609 and GAPDH probes and at 45°C for pEH5 and TmutpEH17 probes. For primer extension, annealing was performed overnight at 17°C, using 10–50 μg of total RNA and 10^5 cpm of ^{32}P-end-labeled primer that was extended by incubation with 400 units of Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL) for 2 hr at 37°C in the reaction buffer recommended by the supplier. The extension products and the protected RNA fragments were analyzed on a 12% polyacrylamide/7 M urea gel. Radioactive RNA fragments of defined length or M13mp8 dideoxy sequencing reaction products were run in parallel as size markers.

RESULTS

Multiple Transcription Start Sites (TSSs) of the cath-D Gene in MCF7 Cells. The human cath-D gene and its 5′ flanking sequences have been previously isolated from an MCF7 genomic library (7). Fig. 1*A* shows the sequence of its promoter region from −200 to +130, taking +1 as the first translated base, with a TATAA sequence located at −48 and a high G+C content (80% in the 200 nucleotides 5′ from the start codon). A cluster of four inverted GC boxes was also found between −135 and −200.

Initiation of transcription from this promoter was then studied in MCF7 cells by RNase protection assay. As shown in Fig. 1*B*, five protected fragments (numbered from I to V) were observed. This heterogeneity in the 5′ end of cath-D transcripts could have been due to different transcription initiation sites or to maturation of a large precursor into smaller forms. However, 5′ end processing is very unlikely, since the longer RNAs persisted in estrogen-stimulated cells after a 12-hr transcription blockade by actinomycin D without being chased by any maturation mechanism (not shown). Using primer extension, we confirmed the position of the two major transcription initiation sites, I and V (Fig. 1*C*). Extended products corresponding to the other initiation sites were also detected on longer autoradiograph exposures. We therefore concluded that in MCF7 cells, cath-D gene transcription was initiated at multiple start sites (TSS) clustered within about 52 bp, and mapped at −20, −44, −51, −60, and −72 bp.

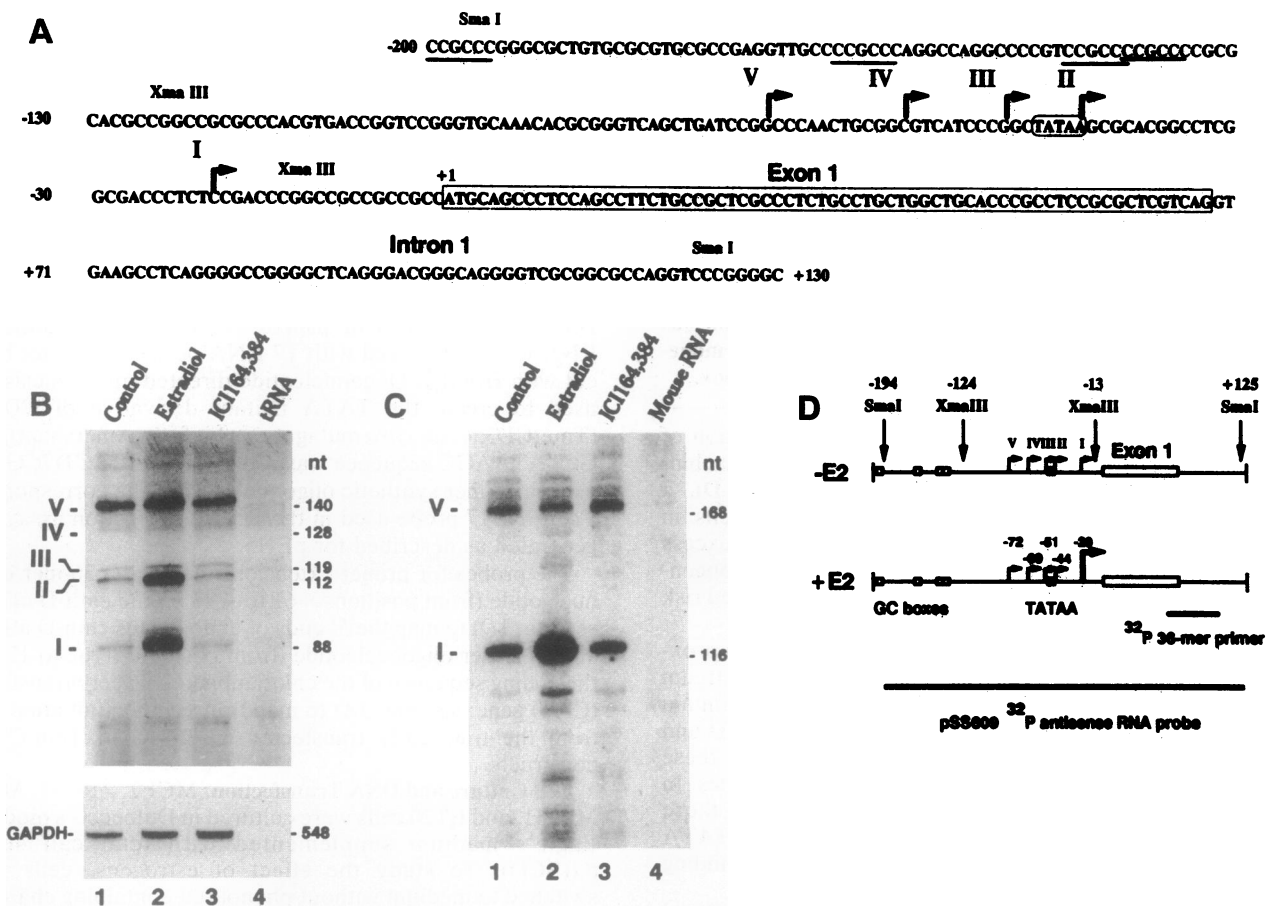

FIG. 1. Localization of cath-D gene transcription start sites in MCF7 cells. (*A*) Proximal promoter region sequence of the human cath-D gene isolated from MCF7 cells. The four GC boxes are underlined. The TATAA sequence (−48/−44) and the first exon are boxed. The five major TSSs (numbered I to V) are indicated by arrows. (*B*) RNase protection assay performed on 10 μg of RNA with the pSS609 RNA antisense probe. Total RNA was extracted from MCF7 cells that had been cultured for 48 hr with 10 nM estradiol, 500 nM pure antiestrogen ICI 164,384, or without ligand as a control. Twenty micrograms of *Escherichia coli* tRNA was used as a control to determine the nonspecific protection of the probe. The autoradiogram shows the five major initiation sites (I to V) with the lengths of the protected fragments in nucleotides (nt). (*C*) Primer extensions performed on the same RNA samples as in *B*. The control for nonspecific extensions (lane 4) was 30 μg of RNA isolated from mouse 3T3 cells. (*D*) Schematic representation of the cath-D promoter region. The GC boxes and the TATAA sequence are represented by squares. The five TSSs (numbered I to V) are represented by arrows and their distances from the +1 nucleotide are indicated. The pSS609 RNA antisense probe and the oligonucleotide primer are also shown.

Estradiol Stimulates Transcription Initiated Only Downstream from the TATA Box. The effect of estradiol and antiestrogen on these start sites was then investigated (Fig. 1 *B* and *C*). Quantification was by the RNase protection assay, corrected by taking into account the different specific activities of the protected fragments and variations in RNA loading, which were estimated by using the constant GAPDH probe analyzed in parallel (Fig. 1*B*). In the absence of estradiol, transcription initiation was approximately identical at the two major sites (TSSI and -V). Initiation at the other TSSs, detectable by RNase protection, represented no more than 30% of total transcription. The same transcription initiation pattern as in the control was observed when MCF7 cells were treated with the pure antiestrogen ICI 164,384 (Fig. 1 *B* and *C*, lanes 3).

As previously described (7), estradiol increased 4-fold the overall cath-D mRNA level. This stimulation was restricted to TSSI, located downstream from the TATA box, which was increased 6- to 10-fold. Transcripts initiated at this site upon estradiol treatment represented, according to the experiments, up to 90% of total cath-D mRNA. No variation was seen for the TSSV site. The slight increase by estradiol of TSSII RNAs species is thought to be an artefact of the RNase protection assay (Fig. 1*B*, lane 2). This stimulation was not detected by primer extension analysis of the same RNA samples (Fig. 1*C*, lane 2) and specifically disappeared after overdigestion of the RNA hybrids by an excess of RNases (not shown).

Fig. 2 shows that in the ZR75-1 breast cancer cell line (lanes 1 and 2), TSSI transcription was estrogen dependent as in MCF7 cells, representing 20% of total transcription in control cells and 90% in stimulated conditions. In the estrogen receptor (ER)-negative cell lines BT20 and MDA-MB231, none of the TSSs were affected by estradiol (Fig. 2, lanes 3–6). The transcription initiation pattern was similar to that of control MCF7 and ZR75-1 cells, but the TSSV-initiated RNA levels were slightly increased. However, in MDA-MB231 cells stably transfected with the HEGO human ER expression vector (clones HC1 and HE5) (10), the TSSI site was stimulated by estradiol (Fig. 2, lanes 7–10) relative to the PB4 clone, which is stably transfected with the vector alone (lanes 11 and 12).

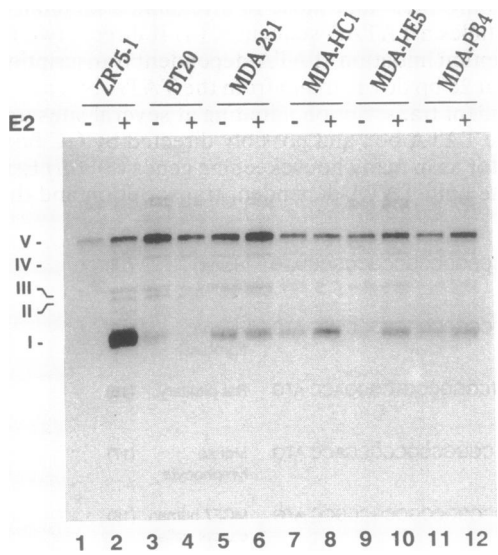

FIG. 2. Transcription initiation sites in different human breast cancer cell lines. The RNase protection assay was performed as described for Fig. 1*B* with 10 μg of total RNA extracted from estrogen-responsive (ZR75-1) or unresponsive (BT20, MDA-MB231) breast cancer cell lines treated (+) or not (−) with estradiol (E2). The assay was also performed with 10 μg of RNA extracted from MDA-MB231 cells stably transfected with the HEGO human ER expression vector (clones MDA-HC1 and MDA-HE5) or with the expression vector alone (clone MDA-PB4) as a control.

Site-Directed Mutagenesis of the TATAA Sequence. To demonstrate the TATA dependence of TSSI-initiated transcription of the cath-D gene, we performed site-directed mutagenesis of the TATA box by substitution of the 5′-CT*CTGC*G-3′ sequence for the wild-type 5′-CTATAAG-3′. The wild-type (CD3510) or the mutated (TmutCD354) constructs (Fig. 3*A*) were transiently transfected into stimulated MCF7 cells with cotransfection of the HEO ER expression vector to increase the efficiency of estrogen (7). As shown in Fig. 3*B* (lane 2), primer extension performed with an oligonucleotide probe complementary to the chloramphenicol acetyltransferase coding sequence revealed that the wild-type CD3510 construct produced several hybrid cath-D–CAT RNAs initiated at TSSI (137 nucleotides) and at the upstream TSSs. The extended fragment of 145 nucleotides corresponded to an additional transcript not detected with the

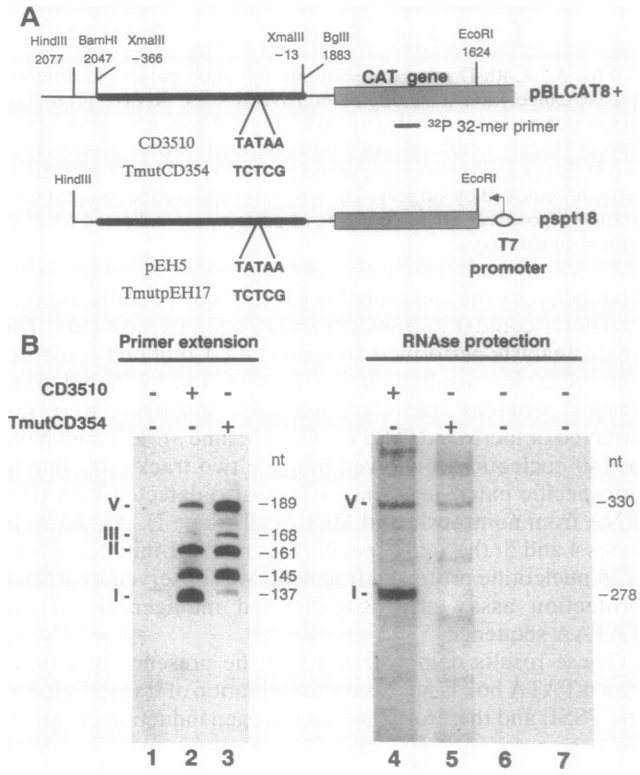

FIG. 3. Site-directed mutagenesis of the TATA box. (*A*) Schematic representation of the two constructs used in transient transfection experiments; the constructs contained the *Xma* III (−366/−13) cath-D promoter fragment (thick line) with the wild-type (CD3510) or the mutated TATA box (TmutCD354) fused to the chloramphenicol acetyltransferase (CAT) gene. The numbers under the names of the restriction sites indicate their position within the pBLCAT8+ sequence or the cath-D promoter. The two corresponding probes pEH5 and TmutpEH17 used in the RNase protection assay and the 32-mer oligonucleotide used for primer extension are represented. (*B*) Subconfluent MCF7 cells plated in 75-cm^2 flasks and cultured in medium supplemented with 10% fetal calf serum were transiently cotransfected by 10 μg of CD3510 (lanes 2 and 4) or TmutCD354 (lanes 3 and 5) and 10 μg of HEO human ER expression vector (lanes 2–5). Total RNA was prepared from two independent transfection experiments, which were analyzed by primer extension (50 μg of RNA) or by RNase protection assay performed on 30 μg of RNA with the pEH5 (lanes 4 and 6) or TmutpEH17 (lanes 5 and 7) probes. The extension products and the protected RNA fragments were resolved on a denaturing 6% polyacrylamide gel. As negative controls, total RNA from nontransfected MCF7 cells were also analyzed (lanes 1, 6, and 7).

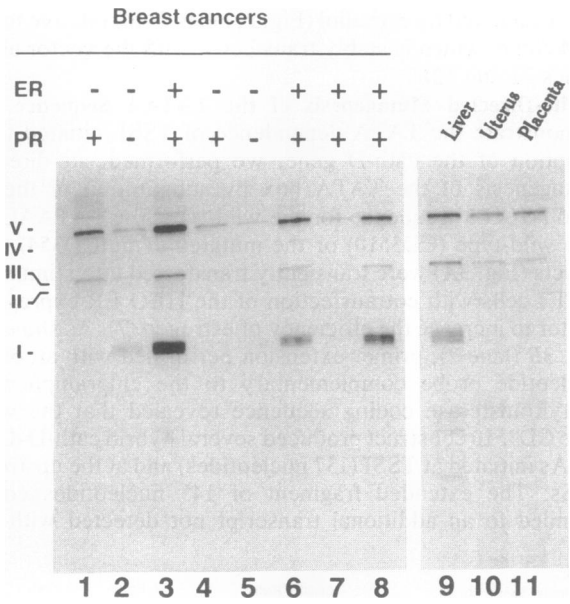

FIG. 4. Cath-D gene transcription initiation pattern in different breast cancer and hormone-responsive tissues. RNase protection assay was performed as described for Fig. 1*B* with RNA from breast cancer biopsy samples (lanes 1–8) or different human tissues (lanes 9–11). Liver and uterus correspond to "normal" tissues adjacent to a tumor removed by surgery. The ER and progesterone receptor (PR) statuses (positive = >10 fmol/mg of protein) are indicated for each breast cancer tissue.

endogenous gene and probably generated by modifications of promoter context in transient transfection experiments. The same analysis performed with the TATA-mutated construct (TmutCD354) resulted in the loss of TSSI-initiated transcripts, whereas the larger extended fragments were not affected or increased (TSSV) (Fig. 3*B*, lane 3). The faint band of 140 nucleotides observed in these two tracks was due to nonspecific extension, since it was also detected with total RNA from nontransfected MCF7 cells (lane 1). As shown in lanes 4 and 5, the same specific loss of TSSI-initiated RNAs (278-nucleotide protected fragment) was observed by RNase protection assay after site-directed mutagenesis of the TATAA sequence.

These results demonstrated that the presence of a functional TATA box is necessary for initiation of transcription at the TSSI, and therefore that the estrogen induction of cath-D mRNA is TATA dependent in estrogen-responsive breast cancer cells.

TATA-Dependent and -Independent Transcription of the cath-D Gene Varies According to Tissue and Breast Cancer Biopsy Samples. In the eight breast cancer tissues analyzed (Fig. 4), transcription initiation patterns corresponding to both control and estradiol-stimulated MCF7 cells were found with two major start sites (TSSI and -V). The four ER-positive tumors (lanes 3 and 6–8) showed a transcription initiation pattern identical to that of stimulated estrogen-responsive breast cancer cell lines, contrasting with the two ER-negative tumors (lanes 1 and 4), with low proportions of TATA-dependent transcription (23% and 38%, respectively) as observed in estrogen-unresponsive breast cancer cell lines. However, predominantly TATA-dependent transcription was also observed in ER- and progesterone receptor-negative tumors (e.g., lane 2) suggesting that factors other than estrogens may also stimulate the level of TATA-dependent transcription. The level of TSSI initiated transcripts was highly correlated ($r = 0.98$, $P < 0.001$) with that of total cath-D mRNA (not shown), suggesting that in breast cancer tumors, overexpression of cath-D mRNA is mostly due to increased TATA-dependent transcription.

The initiation site patterns also varied according to the tissue examined. In human liver, a pattern similar to that of stimulated MCF7 cells was observed (Fig. 4, lane 9), thus suggesting a TATA-regulated transcription. By contrast, the pattern in uterus (at proliferative phase) and placenta (lanes 10 and 11) was similar to that of estrogen-unresponsive breast cancer (mostly TSSV). This is in agreement with a regulation of cath-D in normal endometrium by progesterone but not by estrogens (13).

DISCUSSION

Characterization of the proximal 5′ region of the cath-D gene has given three major pieces of information. (*i*) This promoter has a mixed structure with the general features of housekeeping genes (high G+C content and several potential Sp1-binding sites) and those of a regulated facultative gene that includes a TATAA sequence. (*ii*) It directs two types of transcription initiation: TATA-dependent transcription starting about 28 bp downstream from the TATA box and TATA-independent transcription initiating at several sites upstream from the TATA box and possibly directed by GC boxes and Sp1 factor as in many housekeeping genes (6). (*iii*) Estrogens stimulate only TATA-dependent transcription and therefore

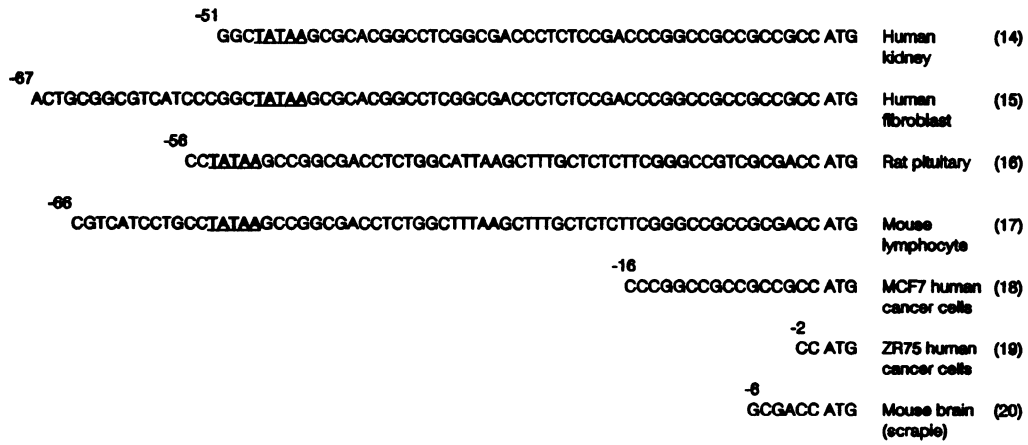

FIG. 5. Comparison of the 5′ lengths of cath-D cDNAs isolated from normal and pathological tissues of different species. The 5′ untranslated sequences of human, rat, and mouse cath-D cDNAs isolated from normal (refs. 14–17) or pathological (refs. 18–20) cells were obtained from the literature as indicated in parentheses. The first codon (+1 corresponding to adenine) is aligned for each sequence and the transcribed TATAA sequence is underlined.

not only increase transcription but also affect the pattern of initiation, with TATA-dependent transcription predominating. This implies that in stimulated conditions, cath-D mRNAs preferentially have short 5′ untranslated sequences, whereas in basal conditions, where TATA-independent transcription is dominant, the proportion of longer cath-D mRNAs is increased.

The prediction of this alternative transcription is that cath-D cDNAs cloned from various tissues and hormonal conditions should have different 5′ ends, containing or not the TATA box. This is indeed the case, as shown in Fig. 5. Sequences of normal cath-D cDNAs were longer in both humans (14, 15) and rodents (16, 17) compared with cDNAs isolated from estrogen-stimulated breast cancer cell lines (18, 19) or from prion-infected cells (scrapie) (20). Moreover, the TATAA sequence was highly conserved at approximately the same position. Such differences in the 5′ length of cath-D mRNA might have biological consequences in terms of stability or initiation of translation and subcellular localization of the protein, for instance (21).

Recently, Redecker *et al.* (22) suggested that the cath-D gene is a typical housekeeping gene lacking a TATA box since, in calcitriol-stimulated monocytes, they detected only an upstream TSS, probably corresponding to TSSV. The discrepancy with our results might be due to different modes of regulation by estrogens and calcitriol.

Since the cath-D gene is controlled by a mixed promoter, this gene has the advantage of being both constitutively expressed from TATA-independent start sites and overexpressed in some physiological (development, tissue remodeling) or pathological (tumor invasion and metastasis) conditions. Promoters of lysosomal enzyme genes generally have housekeeping gene characteristics with no TATA box (for review, see ref. 23). The case of the cathepsin L gene, which is also regulated and overexpressed in cancer cells, appears to be intermediate, since it contains an atypical TATA box and a major initiation site (24). cath-D therefore seems to be the first known example of a lysosomal enzyme gene regulated by a steroid by means of a classical TATA box. Due to its dual promoter, cath-D is intermediate between housekeeping lysosomal enzyme genes with multiple start sites and hormone-regulated tissue-specific genes with a TATA box, such as those coding for the other aspartyl proteases pepsinogen (25) and renin, which is also regulated by a steroid (26). The only known example of a dual promoter with multiple start sites is the metallothionein-I gene, in which initiation involving the TATA box is facilitated by heavy metal inducers and varies according to the tissue (27).

To our knowledge, this is the first report describing a steroid-induced gene with a mixed promoter structure and function, indicating that a steroid hormone displaces transcription initiation from multiple start sites to a predominantly TATA-directed start site. Our results are in agreement with the proposal that steroid receptors stabilize the TATA-controlled preinitiation complex (4, 28, 29). However, some steroid-responsive genes do not contain a TATA box (30, 31), and both the glucocorticoid receptor (32) and the progesterone receptor (33) can also activate transcription of a reporter gene with a mutated nonfunctional TATA box. By contrast, we show that mutation of the human cath-D promoter TATA box inhibits transcription starting at the estrogen-regulated TSSI. Sequences located upstream from the TSSs, able to bind ER and mediate its transcriptional activation, have been characterized (unpublished work). Mechanisms involved in the specific stimulation of TATA-dependent transcription of cath-D gene in its natural context should be determined in further studies.

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