Characterization of Overlapping *XAGE-1* Transcripts Encoding a Cancer Testis Antigen Expressed in Lung, Breast, and Other Types of Cancers

Kristi A. Egland, Vasantha Kumar, Paul Duray, and Ira Pastan[1]

Laboratory of Molecular Biology [K. A. E., V. K., I. P.], and Laboratory of Pathology [P. D.], National Cancer Institute, NIH, Bethesda, Maryland 20892

Abstract

Cancer testis (CT) antigens have an expression pattern that is predominantly restricted to testis in normal tissues, yet they are expressed in many different histological types of cancers. One previously described member of the CT antigen family, XAGE-1, was shown to be expressed in Ewing's sarcomas and rhabdomyosarcomas. Here we show that *XAGE-1* is also expressed in breast cancer, prostate cancer, and different types of lung cancers, including lung squamous cell carcinoma, adenocarcinoma, small cell lung carcinoma, and non-small cell lung carcinoma. In addition, *XAGE-1* mRNA was present in ovarian cancer, melanoma, glioblastoma, T-cell lymphoma, chronic myelogenous leukemia, and histiocytic lymphoma cell lines. We also characterized the *XAGE-1* transcript by primer extension analysis and found that transcription of the *XAGE-1* gene is initiated from two distinct start sites, resulting in two overlapping transcripts, *XAGE-1a* and *XAGE-1b*. *XAGE-1a* contains two in-frame ATG translational start codons; whereas *XAGE-1b* initiates downstream of the first ATG start codon. Our results suggest that *XAGE-1b* is the dominant transcript, and that translation begins with the second ATG start codon, producing a 9 kDa protein. Because *XAGE-1* is expressed in such a diverse range of cancers, it has potential to be used as a target for many cancer immunotherapies.

Introduction

XAGE-1 is a member of the family of CT[2] antigens. The expression of CT antigens in normal tissues is predominantly restricted to testis, with expression in the spermatogonia cells (1). However, CT antigens are expressed by many different types of human cancers, such as melanomas, head and neck carcinomas, lung tumors, bladder carcinomas, and breast cancers, which makes them attractive targets for cancer immunotherapy (reviewed in Refs. 2–4). CT antigens are rarely expressed in leukemias, lymphomas, or colorectal tumors. Several members of the CT antigen family, such as MAGE (5), BAGE (6), and GAGE (7), were initially identified as targets for cytotoxic T cells in a patient with melanoma. Others, such as HOM-MEL-40/SSX2 (8), NY-ESO-1 (9), and SCP1 (10) were discovered by using a serological approach, referred to as SEREX analysis, in which patient sera are used as the source of antibody to screen human recombinant tumor cDNA expression libraries. *PAGE4* was identified by a computer-based screening strategy that searched the EST database for clusters of ESTs that are preferentially expressed in prostate cancers (11). *XAGE-1* was discovered by "homology walking" using the *PAGE4* sequence (12). The *XAGE-1* cluster contains ESTs from testis, alveolar rhabdomyosarcoma, Ewing's sarcoma, and germ-cell tumor cDNA libraries. The predicted XAGE-1 protein shares strong homology with members of the GAGE/PAGE family in the COOH terminus (13).

Like most CT antigen family members, the *XAGE-1* gene is located on the X chromosome (13). In normal tissues, *XAGE-1* is highly expressed in testis. Very weak expression is detected in lung and peripheral blood lymphocytes by RT-PCR analysis but not by RNA blotting techniques (13). Consistent with the EST database prediction, *XAGE-1* is strongly expressed in Ewing's sarcomas and alveolar rhabdomyosarcomas (13). Because CT antigens are expressed in a wide range of cancers, we analyzed numerous cancer cell lines as well as many patient tumor samples for the expression of *XAGE-1*. Here, we report that *XAGE-1* is highly expressed in breast cancer, prostate cancer, and many types of lung cancers, including squamous cell carcinoma, small cell carcinoma, non-small cell carcinoma, and adenocarcinoma.

Prompted by the strong expression of *XAGE-1* in tumors of different origins, we further characterized the *XAGE-1* transcript, which is composed of four exons and contains two in-frame ATG translational start codons. The longest ORF consists of 146 amino acid residues encoding a putative protein with a molecular mass of 16.3 kDa. The second ATG lies 193 bp downstream from the first translational start codon. Translation from the second start codon would yield a 9 kDa protein. The 16.3 kDa protein contains a hydrophobic region in the NH_2 terminus, which is missing in the 9 kDa polypeptide. Here, we report the results of a primer extension analysis that shows two distinct start sites for the *XAGE-1* gene. The second transcriptional start site is located downstream of the first translational start codon, and, therefore, the shorter transcript would encode only the smaller protein.

Received 12/19/01; revised 2/25/02; accepted 3/12/02.

[1] To whom requests for reprints should be addressed, at Laboratory of Molecular Biology, National Cancer Institute, NIH, 37 Convent Drive MSC 4264, Room 5106, Bethesda, MD 20892-4264. Phone: (301) 496-4797; Fax: (301) 402-1344; E-mail: pasta@helix.nih.gov.

[2] The abbreviations used are: CT, cancer-testis; EST, expressed sequence tag; RT-PCR, reverse transcription-PCR; ORF, open reading frame; poly(A), polyadenylation; SCLC, small cell lung carcinoma; NSCLC, non-SCLC; ER, estrogen receptor; RACE, rapid amplification of cDNA ends; CMV, cytomegalovirus.

Downloaded from http://aacrjournals.org/mct/article-pdf/1/7/441/2317761/gd0702000441.pdf by guest on 10 October 2024

The shorter transcript appears to be more abundant than the longer one. *In vitro* transfection experiments consistently show that translation of *XAGE-1* begins with the second ATG in the reading frame, producing a 9 kDa protein.

Materials and Methods

Primers. The primers used are the following: Xa-5′-For (5′-GGACCTGGGAAGGAGCATAG-3′); Xa-5′-Rev (5′-GACGC-CCAGTGAACATGCG-3′); Xa-3′-For (5′-TCAGTCAAACACC-GGGGATAAATC-3′); Xa-1 (5′-CAGCTTGTCTTCATTTAAACT-TGTGGTTGC-3′); Xa-2 (5′-TCCCAGGAGCCCAGTAATGGA-G-3′); Xagen-forward (5′-CCTCAGCCTCCCGAGTAGC-3′); Xagext.1 (5′-CCGCCGTGTCTCAGTAGC-3′); Xagext.3 (5′-CCGCCGTGTCTCAGTAGCGG-3′); Xagext.4 (5′-CAAAGGAG-CATGCGCAGTGAGG-3′); Xa-CMV-F (5′-AATCCG*AAGCTT*C-ACGAGGGAACCTCACG-3′); Xa-CMV-R (5′-AATCCG*CTCG-AG*TTAAACTTGTGGTTGCTCTTC-3′); Xa-2ATT-F (5′-GGAGC-CCAGTAATTGAGAGCCCCAAAAAG-3′); Xa-2ATT-R (5′-TTT-GGGGCTCTCAATTACTGGGCTCCTG-3′); Xa-11 (5′-GCTC-CTTTGGTGCCCACCTC-3′); Xa-12 (5′-CTTTCTCCGCTACT-GAGACACGG-3′); Xa-His-F (5′-CAGGCTCGAGATGCTCCTT-TGGTGCCCAC-3′); Xa-His-R (5′-CAGGCCATGGTTAAACTT-GTGGTTGCTCTTCAC-3′). The primers were synthesized by Lofstrand Labs Ltd. (Gaithersburg, MD). The primers Ori-Actin-F (5′-GCATGGGTCAGAAGGAT-3′) and Ori-Actin-R (5′-CCAATGGTGATGACCTG-3′) were purchased from OriGene Technologies (Rockville, MD).

Constructs. The pCRII-XAGE plasmid contains a 592-bp *XAGE-1* DNA insert spanning the region 83 bp upstream from the first ATG translational start codon to the first poly(A) signal sequence. This DNA fragment was cloned into pCRII-TOPO (Invitrogen, Carlsbad, CA) using the TOPO TA cloning system (Invitrogen) according to the manufacturer's manual (13). The plasmid, pXa-gen, contains a 518-bp fragment that was amplified from genomic DNA using a primer, Xagen-For, that anneals 276 bp upstream from the *XAGE-1a* start of transcription (Fig. 1*A*), and primer Xagext.1 (Fig. 1*A*). This fragment spans a region 276 bp upstream from *XAGE-1a* to the 3′ end of exon 1. This fragment was amplified with Platinum Pfx DNA polymerase (Life Technologies, Inc., Rockville, MD) and cloned into pCRII-TOPO (Invitrogen) using the TOPO TA cloning system (Invitrogen) according to the manufacturer's manual. For *in vitro* transfection, a DNA fragment including the entire *XAGE-1a* ORF and 21 bp 5′ of the first putative ATG start codon was amplified by PCR using primers Xa-CMV-F and Xa-CMV-R and cloned into the *Hin*dIII and *Xho*I sites of pcDNA3 (Invitrogen) to allow expression from the CMV promoter. The resulting plasmid is designated as pCMV-XAGE. The second ATG in the *XAGE-1* ORF was changed to ATT by using the QuikChange Site-Directed Mutagenesis kit using primers Xa-2ATT-F and Xa-2ATT-R according to the manufacturer's instructions (Stratagene, La Jolla, CA). This plasmid is referred to as pXAGE-2-ATT. The construct pHis-XAGE was generated by PCR amplifying the entire ORF of *XAGE-1a* (Fig. 1*A*) using primers Xa-His-F and Xa-His-R and cloning it into the *Xho*I and *Nco*I sites of pET3 (Novagen, Madison, WI). All inserts were confirmed by DNA sequencing.

Cell Culture. LNCaP and TC71 cell lines were maintained in RPMI 1640 (Quality Biologicals, Gaithersburg, MD) with 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin/streptomycin, and either 10 FBS or 10% heat inactivated-FBS, respectively. MDA-MB-231 and 293T cells were maintained in DMEM (Quality Biologicals) with 2 mM L-glutamine, penicillin/streptomycin, and either 5 or 10% FBS, respectively. The cells were maintained at 37°C with 5% CO_2.

Cancer-profiling Array and Northern Blot Hybridization. The cancer profiling array was purchased from Clontech (Palo Alto, CA). The 414-bp *XAGE-1* probe used for hybridization was generated by digesting the EST clone af89d01.s1 with *Eco*RI and *Not*I (see Fig. 1*B*, *Probe D*). The probe was labeled with ^{32}P by random primer extension (Lofstrand Labs Ltd.), and the hybridization conditions were provided as described previously (13).

Samples for Northern blot hybridization, 2 μg of poly(A) RNA per lane, were separated using a 1.5% agarose gel containing 2% formaldehyde. TC71 poly(A) RNA was generated using the FastTrack 2.0 mRNA isolation system from Invitrogen (Carlsbad, CA). For use as probes, the 130-bp *XAGE-1* fragments were amplified using either primers Xa-5′-For and Xa-5′-Rev with pXa-gen as a template (Fig. 1*B*, *Probe A*) or primers Xa-3′-For and Xa-1 with pCRII-XAGE as a template (Fig. 1*B*, *Probe B*). The probes were labeled with ^{32}P by random primer extension (Lofstrand Labs Ltd.). The hybridization and washing conditions were performed as described previously (13). The 0.16–1.77-kb RNA ladder was purchased from Life Technologies, Inc. (Rockville, MD).

RT-PCR Analysis. Total RNA was isolated from frozen tumor samples acquired from the Cooperative Human Tissue Network and tissue culture cell lines using the StrataPrep Total RNA Miniprep Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Isolation of poly(A) RNA from tissue culture cells was performed using the FastTrack 2.0 mRNA isolation system from Invitrogen (Carlsbad, CA). Human testis total RNA was purchased from Clontech (Palo Alto, CA). To generate single-stranded cDNAs, either total RNA (4 μg) or poly(A) RNA (100 ng) was used with Superscript II RT (Life Technologies, Inc.) and oligo(dT) priming as described previously (14). PCR reactions were performed using the following protocol: initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 2 min with a final 5 min extension at 72°C. Similar conditions (14) were used with the human breast cancer Rapid-Scan gene expression panel (OriGene Technologies).

Primer Extension Analysis. Total RNA was isolated from the Ewing's sarcoma cell line, TC71, by the StrataPrep Total RNA Miniprep kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Testis total RNA was purchased from Clontech. The primers Xagext.3 and Xagext.4 were end-labeled by Lofstrand Labs Ltd. The ^{32}P-labeled primers (950,000 dpm for Xagext.3 and 1,503,000 dpm for Xagext.4) were each added to 20 μg of total RNA. The template and primers were annealed at 70°C for 10 min and immediately placed on ice. In a final volume of 10 μl, 100 units of Superscript II reverse transcriptase (Life Technologies, Inc), 250 μM dNTPs, 2 mM DTT, and 8 units of RNasin (Roche Molecular

Downloaded from http://aacrjournals.org/mct/article-pdf/1/7/441/2317761/gd0702000441.pdf by guest on 10 October 2024

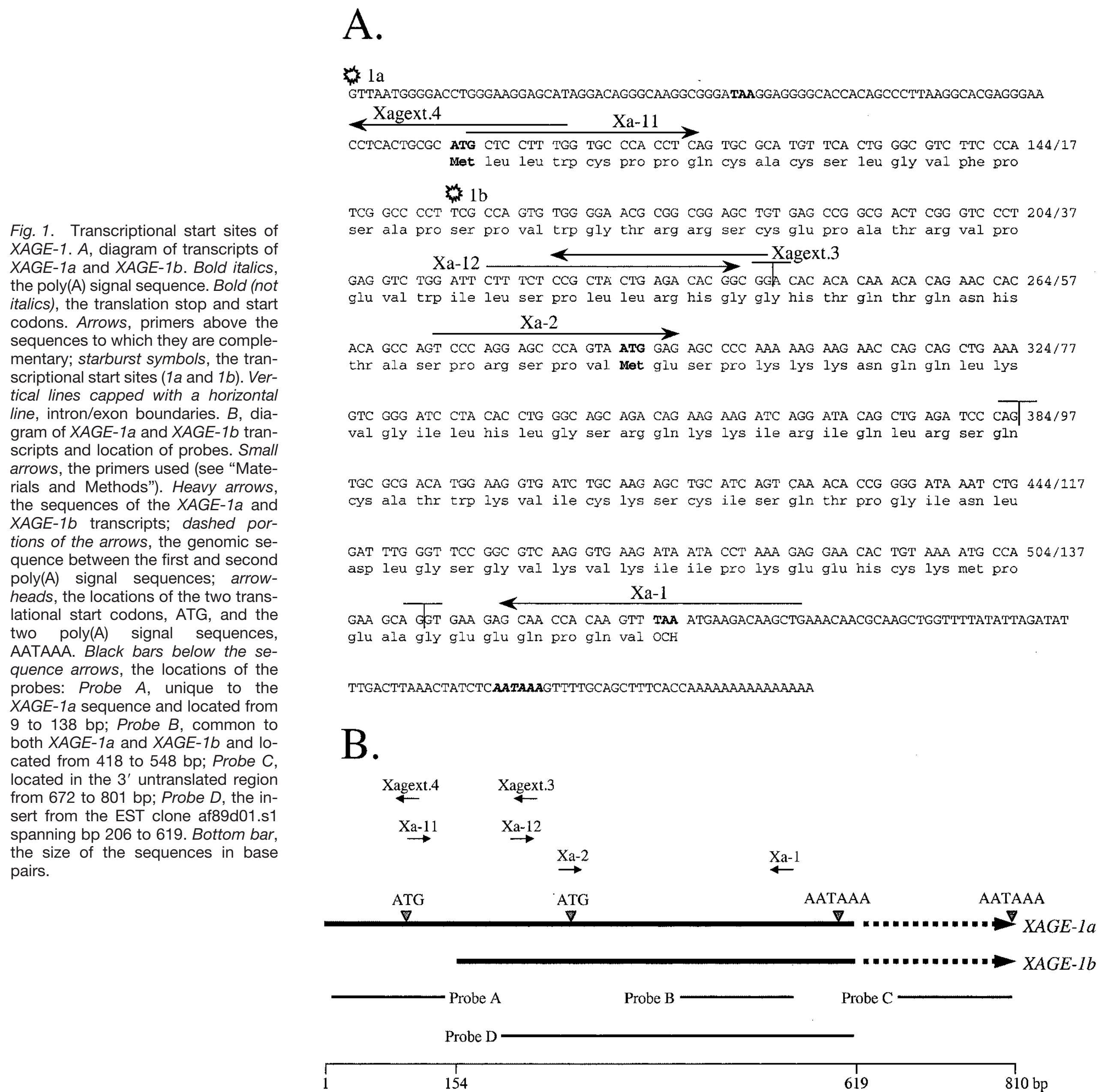

Fig. 1. Transcriptional start sites of *XAGE-1*. *A*, diagram of transcripts of *XAGE-1a* and *XAGE-1b*. *Bold italics*, the poly(A) signal sequence. *Bold (not italics)*, the translation stop and start codons. *Arrows*, primers above the sequences to which they are complementary; *starburst symbols*, the transcriptional start sites (*1a* and *1b*). *Vertical lines capped with a horizontal line*, intron/exon boundaries. *B*, diagram of *XAGE-1a* and *XAGE-1b* transcripts and location of probes. *Small arrows*, the primers used (see "Materials and Methods"). *Heavy arrows*, the sequences of the *XAGE-1a* and *XAGE-1b* transcripts; *dashed portions of the arrows*, the genomic sequence between the first and second poly(A) signal sequences; *arrowheads*, the locations of the two translational start codons, ATG, and the two poly(A) signal sequences, AATAAA. *Black bars below the sequence arrows*, the locations of the probes: *Probe A*, unique to the *XAGE-1a* sequence and located from 9 to 138 bp; *Probe B*, common to both *XAGE-1a* and *XAGE-1b* and located from 418 to 548 bp; *Probe C*, located in the 3′ untranslated region from 672 to 801 bp; *Probe D*, the insert from the EST clone af89d01.s1 spanning bp 206 to 619. *Bottom bar*, the size of the sequences in base pairs.

Downloaded from http://aacrjournals.org/mct/article-pdf/1/7/441/2317761/gd0702000441.pdf by guest on 10 October 2024

Biochemicals, Indianapolis, IN) was added, and the reactions were allowed to proceed for 30 min at 42°C. An equal volume of loading dye (98% formamide, 10 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue) was added to stop the reactions. After heating the reactions to 95°C for 5 min, the primer extension products were separated on an 8% polyacrylamide-urea gel. Sequencing ladders were generated using the *fmol* DNA sequencing system (Promega, Madison, WI) with the primers described above and the plasmid, pXa-gen.

***In Situ* Hybridization.** The plasmids, pCRII (Invitrogen) and pCRII-XAGE, were labeled using the BioNick Labeling System (Life Technologies, Inc. Rockville, MD) as described previously (15). Biotinylated pCRII without any insert was used as a negative control. The slides were hybridized using the *in situ* Hybridization and Detection system (Life Technologies, Inc.) following the vendor's recommendation with a few modifications, as described previously (16). The slides were counterstained using 0.2% Light Green stain, rinsed through a series of alcohol grades, and mounted in Cytoseal. They were photographed at ×100 with a digital camera mounted on a Nikon Eclipse E800 microscope.

Generation of Polyclonal Antibodies against XAGE-1. Polyclonal antibodies were generated against a *Pseudomonas* exotoxin (ΔPE)-XAGE fusion protein as described previously (17). Briefly, a ΔPE-XAGE fusion protein was made by

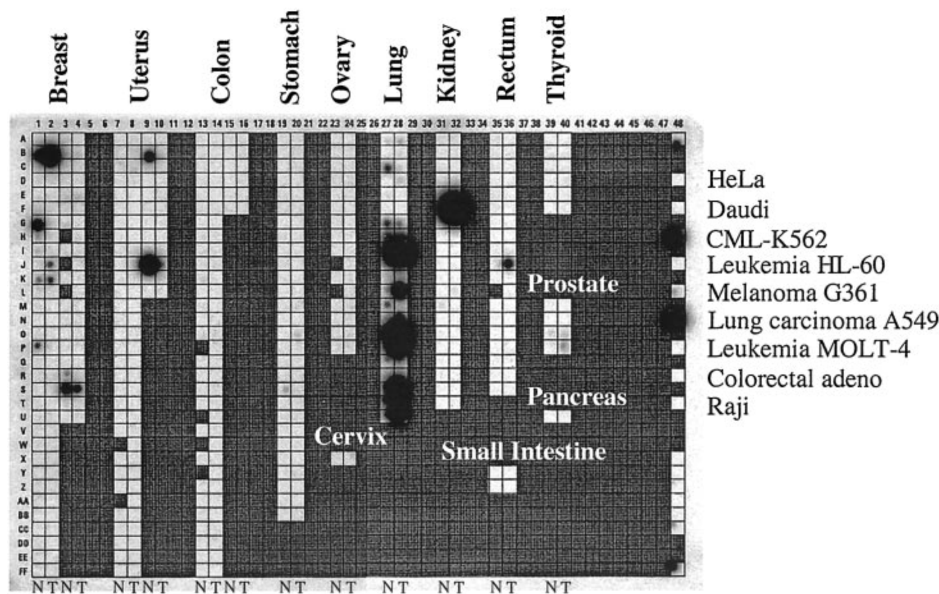

Fig. 2. Expression of *XAGE-1* in matched normal and tumor tissues. The cancer profiling array contains cDNA samples from 241 tumor and corresponding normal tissues from individual patients, as well as cDNA samples from 9 different cell lines (Clontech). The blot was probed with a 414 bp *XAGE-1* cDNA probe derived from the EST clone af89d01.s1 (Fig. 1*B*, *Probe D*). The tumor types are indicated *above and on the blot*. *On the right*, the identification of each tumor cell line. *N*, the columns of individual normal tissue samples; *T*, the columns derived from tumor tissue samples. The blot was exposed to film for 6 days.

cloning a 3′ *XAGE-1* fragment encoding the 109 COOH-terminal residues of XAGE-1 in frame with the 3′ end of a mutant ΔPE gene containing a single codon deletion that renders the encoded enzyme catalytically inactive. The ΔPE-XAGE protein was overexpressed in *Escherichia coli* BL21(λDE3), and inclusion bodies containing the fusion protein were isolated and washed. Female white New Zealand rabbits were immunized with the purified inclusion bodies. The antiserum from the rabbits was purified over a protein A column followed by an immobilized *E. coli* lysate column, according to the manufacturer's instructions (Pierce, Rockford, IL).

***In Vitro* Transfection.** Human embryonic kidney cells, 293T, were transfected with pcDNA3, pCMV-XAGE, or pXAGE-2-ATT by the protocol according to Pear *et al.* (18). Briefly, 293T cells were transfected by using $CaPO_4$ precipitation. The cells were harvested 48 h posttransfection, and whole-cell protein extracts were prepared from cells containing vector only, pCMV-XAGE, pXAGE-2-ATT, or untransfected cells. A Western-immunoblot analysis was performed on the protein extracts. Whole-cell protein extracts (30 μg) and 100 ng of recombinant His-XAGE were run on a 16.5% polyacrylamide gel (Bio-Rad) and transferred to a polyvinylidene fluoride membrane (Millipore). The membranes were probed with 10 μg/ml of either pre-bleed or ΔPE-XAGE antiserum. A chemiluminescence Western blotting kit was used to detect XAGE-1 on the membrane according to the manufacturer's instructions (Roche Molecular Biochemicals).

Results

***XAGE-1* Is Expressed in Tumors of Various Histological Types.** Because other members of the CT antigen family are expressed in a wide range of cancers (3), we were stimulated to examine the range of cancers that express *XAGE-1*. A cancer-profiling array (Clontech, Palo Alto, CA) containing 241 matched normal and tumor cDNA samples from individual patients was analyzed for *XAGE-1* expression using a 414-bp insert of the EST af89d01.s1 as a probe (Fig. 2; see Fig. 1*B*, *Probe D*). *XAGE-1* expression was detected in two breast lobular carcinomas (Fig. 2, *2B* and *2K*) and two breast infiltrating ductal carcinomas (Fig. 2, *2J* and *4S*). *XAGE-1* expression was also detected in six normal breast tissue samples (Fig. 2, *1B*, *1G*, *1K*, *1P*, *3R*, and *3S*). *XAGE-1* was abundantly expressed in numerous lung carcinomas, including seven squamous cell carcinomas (Fig. 2, *28A*, *28C*, *28D*, *28G*, *28Q*, *28T*, and *28U*), three adenocarcinomas (Fig. 2, *28I*, *28N*, and *28O*), and two bronchiolo-alveolar adenocarcinoma (Fig. 2, *28L* and *28P*). In addition, *XAGE-1* was expressed in two samples of normal uterus (Fig. 2, *9B* and *9J*), one kidney transitional cell carcinoma (Fig. 2, *32F*), one rectal adenosquamous carcinoma (Fig. 2, *36J*). Weak expression of *XAGE-1* was observed in a prostate adenocarcinoma (Fig. 2, *40P*). The human cancer cell lines, chronic myelogenous leukemia K562 and lung carcinoma A549, both express *XAGE-1*. Expression of *XAGE-1* was not detected in colon, stomach, ovary, cervix, thyroid, pancreas, or small intestine in either the normal or the cancer tissue samples. To verify the integrity of the cDNA on the array, the cancer profiling array was stripped and probed with ubiquitin. All of the cDNA samples on the array hybridized with the ubiquitin probe (data not shown).

***XAGE-1* Expression in Breast and Prostate Cancer.** To further investigate the expression of *XAGE-1* in normal breast tissue and breast cancer, we conducted a RT-PCR analysis using a human breast cancer rapid-scan panel (OriGene Technologies) containing 12 normal breast tissue cDNA samples and 12 breast tumor cDNA samples derived from 24 individual patients (Fig. 3). The PCR reactions were performed using primers Xa-1 and Xa-2 (Fig. 1*B*). Five of the 12 normal breast samples contained a very faint 273-bp fragment, which indicated that the normal breast samples expressed *XAGE-1* either weakly or not at all (Fig. 3*A*). A strong

Downloaded from http://aacrjournals.org/mct/article-pdf/1/7/441/2317761/gd0702000441.pdf by guest on 10 October 2024

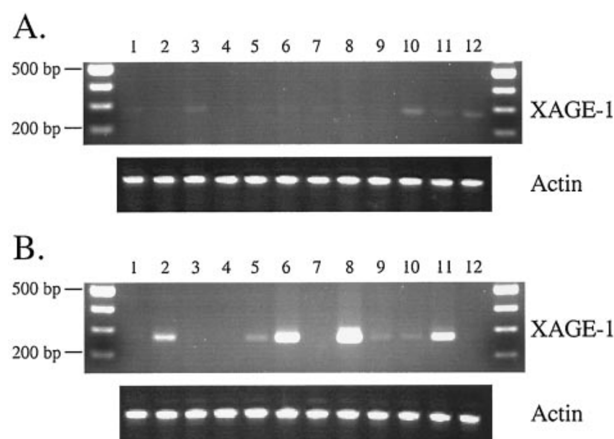

Fig. 3. Expression of *XAGE-1* in normal human breast tissue and breast cancer. PCR reactions were performed using a Human Breast Cancer Rapid-Scan panel (OriGene Technologies) containing cDNA samples derived from 24 different patients. The panel contains (*A*) 12 cDNA samples derived from normal breast and (*B*) 12 cDNA samples derived from breast tumors. PCR reactions were performed using primers Xa-1 and Xa-2 to the *XAGE-1* gene. These primers are located in different exons and amplify a 273-bp product (see Fig. 1*A*). PCR reactions using actin primers Ori-actin-F and Ori-actin-R were performed separately and produced a 640-bp product. The PCR products were analyzed by agarose gel electrophoresis with ethidium bromide staining.

273-bp fragment was detected in 4 of the 12 breast cancer cDNA samples and a weak band in three other samples (Fig. 3*B*). Compared with the actin signal, which was strong in all of the samples, the PCR products from the breast tumor samples, when present, were more abundant than were the products from the normal samples. Although RT-PCR analysis is semiquantitative, this result suggested that *XAGE-1* might be up-regulated in breast cancer.

In situ hybridization using *XAGE-1* as a probe was performed on tissue sections from normal breast and two cases of breast carcinoma with two histological subtypes, infiltrating ductal and infiltrating lobular carcinoma, respectively (Fig. 4, *A* and *B*). The normal breast sections showed weak expression of *XAGE-1*, whereas the signal in the breast tumors was very intense. In addition, tissue sections from two cases with both normal prostate and adenocarcinoma with two histological subtypes, lobular cribriform and acinar carcinoma, respectively, were examined (Fig. 4, *C* and *D*). The normal prostate sections showed very weak signal in the epithelial cells, and the prostate cancer cells showed a moderately intense signal for *XAGE-1* (Fig. 4, *C* and *D*). As a negative control, pCRII containing no insert was used as a probe for the breast and prostate tissue sections, and no signal was detected.

***XAGE-1* Is Expressed in Many Types of Lung Cancers.** The results from the cancer-profiling array indicate that *XAGE-1* is expressed in lung squamous cell carcinomas and lung adenocarcinomas (Fig. 2). To corroborate these results, total RNA was isolated from frozen tumor samples from patients and subjected to RT-PCR analysis using primers to *XAGE-1* (Table 1). *XAGE-1* was expressed in two of three lung squamous cell carcinomas and two of three lung adenocarcinomas. Other CT antigen genes, such as *MAGE*, *BAGE*, and *GAGE*, were expressed in a significant proportion of NSCLCs (3, 19). In addition, *NY-ESO-1* is expressed in both NSCLCs and SCLCs (20). To address whether *XAGE-1* is also expressed in NSCLC and SCLC, total RNA was isolated from frozen tumor samples, and *XAGE-1* expression was determined by RT-PCR analysis using primers to *XAGE-1* (Table 1). *XAGE-1* was expressed in all three of the SCLC samples analyzed and in both of the NSCLC samples. Similar to other CT antigens, *XAGE-1* is expressed in NSCLCs and SCLCs.

***XAGE-1* Expression in Human Cancer Cell Lines.** To further characterize *XAGE-1* expression in various cancer types, we surveyed 23 different human cancer cell lines (Table 2). *XAGE-1* expression was examined by RT-PCR analysis using PCR primers Xa-1 and Xa-2 to the *XAGE-1* gene. To compare relative levels of *XAGE-1* expression between cell lines, separate PCR reactions were performed using primers to β-actin to verify the quality of the generated cDNA (data not shown). Expression of *XAGE-1* was not detected in any of the assayed ER-positive breast cancer cell lines, ZR-75–1, MCF7, and BT-474 (Table 2). However, the ER-negative breast cancer cell lines, MDA-MB-231, SK-BR-3, and MDA-MB-468, all expressed *XAGE-1*. Certain tumor types, such as gastrointestinal carcinomas, colorectal carcinomas, renal cancers, leukemias, and lymphomas, rarely express CT antigens (3, 19, 21). Similarly, *XAGE-1* is not expressed in any of the studied colon, rectum, colorectal, Burkitt's lymphoma, or neuroblastoma cancer cell lines. However, *XAGE-1* is expressed in the T-cell lymphoma cell line, HUT102, and in the histiocytic lymphoma cell line, U937, which is rare for CT antigens.

***XAGE-1* Has Two Transcriptional Start Sites.** Previously, RACE-PCR was used to determine the 5′ end of the *XAGE-1* transcript (13). The longest RACE-PCR product corresponded to a guanine located 85 bp upstream from the first potential ATG translational start codon. To further verify the transcription initiation start site of *XAGE-1*, a primer extension analysis was performed using total RNA isolated from TC71, a Ewing's sarcoma cell line, and normal testis; both of which were previously shown to express *XAGE-1* (13; Fig. 5). The primer used, Xagext.3, is located in the first exon of *XAGE-1* to ensure that the primer extension product properly aligns with the DNA sequencing ladder, which was cloned from genomic DNA (Fig. 1*A*). Surprisingly, the primer extension product derived from the Xagext.3 primer corresponds to a transcription initiation start site located 58 bp downstream from the first ATG translational start codon (Fig. 5*B*). To map the 5′-most transcriptional start site of *XAGE-1*, the primer Xagext.4 was used. The most abundant primer extension product derived from the Xagext.4 primer corresponds to a guanine located 8 bp upstream from the start of the longest RACE-PCR product reported previously (Figs. 1*A* and 5*A*; Ref. 13). This primer extension analysis revealed that there are two distinct starts sites for the *XAGE-1* transcript. The 5′-most *XAGE-1* transcript will be referred to as *XAGE-1a* and the downstream transcript will be referred to as *XAGE-1b*. With the poly(A) tails excluded, the *XAGE-1a* transcript was 619 bp in length and *XAGE-1b* was 466 bp.

Downloaded from http://aacrjournals.org/mct/article-pdf/1/7/441/2317761/gd0702000441.pdf by guest on 10 October 2024

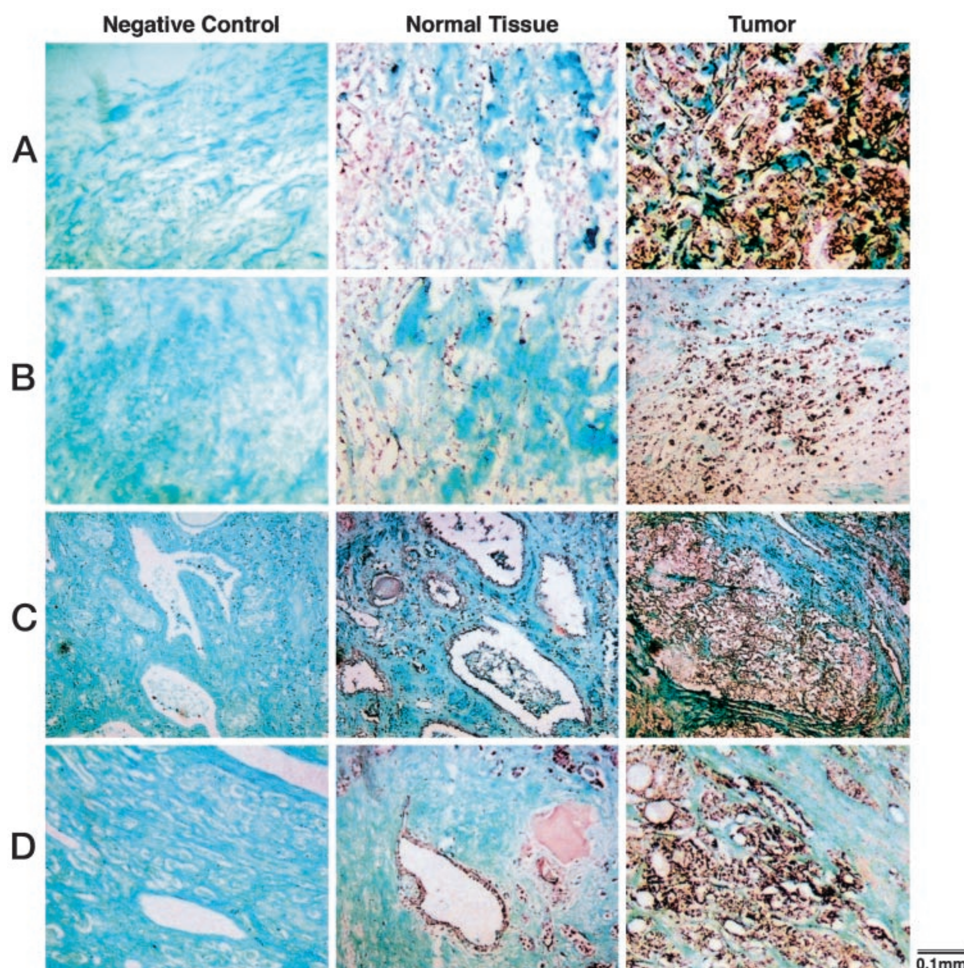

Fig. 4. *In situ* hybridization analysis of *XAGE-1* expression in breast and prostate cancers. *A* and *B*, breast tissue sections from two different patients. The tumor sections in *A* and *B* are infiltrating ductal and infiltrating lobular carcinomas, respectively. Increased expression of *XAGE-1* in tumor *versus* benign is visible in *A* and *B*. *C* and *D*, prostate tissue sections from two different patients. The prostate tumor in *C* is a Gleason grade 4 lobular cribriform type adenocarcinoma. The adenocarcinoma in *D* represents an infiltrating acinar carcinoma, Gleason grade 3–4. Increased expression of *XAGE-1* in prostate carcinoma is seen in both C and D. The negative control slides were probed with biotinylated pCRII without any insert. The normal breast and breast tumor sections, as well as the normal prostate and prostate tumor sections were probed with biotinylated pCRII-XAGE.

Downloaded from http://aacrjournals.org/mct/article-pdf/1/7/441/2317761/gd0702000441.pdf by guest on 10 October 2024

Table 1 Expression of *XAGE-1* in lung cancers

Total RNA was isolated from frozen tumor samples, and expression levels were determined by RT-PCR using primers Xa-1 and Xa-2 to the *XAGE-1* gene (see Fig. 1*A*). These primers are located in different exons and amplify a 273-bp product. The RNA templates were checked for integrity using the Agilent 2100 Bioanalyzer, and separate PCR reactions were performed using actin primers to confirm the quality of the generated cDNA.

Lung cancer type	Total no. tested	No. of cancers that express *XAGE-1*	% of samples expressing *XAGE-1*
SCLC	3	3	100
NSCLC	2	2	100
Squamous cell carcinoma	3	2	67
Adenocarcinoma	3	2	67

Both start sites were present in RNA derived from normal testis and the Ewing's sarcoma cell line.

Relative Abundance of the Two *XAGE-1* Transcripts. The primer extension analysis shows that the *XAGE-1* gene has two transcriptional start sites (Fig. 5). The *XAGE-1a* transcript encodes both of the putative 16 kDa and 9 kDa proteins. Because the start site of *XAGE-1b* is located downstream from the first ATG translational start codon, this transcript encodes the 9 kDa protein only. To gain information about the relative abundance of the two transcripts, RT-PCR analysis was performed using primer Xa-1 and one of two different 5′ primers, Xa-11 or Xa-12 (Fig. 1). One 5′ primer, Xa-11, is unique to *XAGE-1a*; whereas primer Xa-12 will anneal to cDNA derived from both transcripts (see Fig. 1*B*). PCR reactions were conducted using cDNAs derived from testis, TC71, LNCaP, and MDA-MB-231 as templates. After 35 cycles, PCR reactions performed with primers Xa-11 and Xa-1 produced a weak 453-bp product in testis and TC71, whereas no products were observed with LNCaP or MDA-MB-231 (Fig. 6*A*). To verify the quality of the cDNA templates, separate PCR reactions were performed using actin primers (Fig. 6*A*). In addition, as a positive control for primers Xa-11 and Xa-1, a PCR reaction was performed using pCRII-XAGE as the template, and an abundant product was observed (Fig. 6*B*). In contrast to the results seen with primers Xa-11 and Xa-1, an extremely abundant 332-bp product was observed with all of the cDNA samples using primers Xa-12 and Xa-1 (Fig. 6*A*). These results suggest that *XAGE-1b* is more abundant than the *XAGE-1a* transcript.

To further quantify the abundance of the two *XAGE-1* transcripts, a Northern blot analysis was performed using

Table 2 Expression of *XAGE-1* in human cancer cell lines

Either total or poly(A) RNA was isolated from the tumor cell lines. Expression levels were determined by RT-PCR using Xa-1 and Xa-2 primers to the *XAGE-1* gene (see Fig. 1*A*). These primers are located in different exons and amplify a 273-bp product. Separate PCR reactions were performed using actin primers to verify the quality of the generated cDNA.

Cell line	Cancer type	Level of *XAGE-1* expression
LNCaP	Prostate	Strong
PC3	Prostate	None
DU145	Prostate	Strong
ZR-75-1	Breast	None
MCF7	Breast	None
BT-474	Breast	None
MDA-MB-231	Breast	Strong
SK-BR-3	Breast	Weak
MDA-MB-468	Breast	Weak
OVCAR	Ovarian	Strong
FEM-X	Melanoma	Weak
HUT102	T cell lymphoma	Weak
U937	Histiocytic lymphoma	Strong
Daudi	Burkitt's lymphoma	None
JD38	Burkitt's lymphoma	None
Raji	Burkitt's lymphoma	None
A-172	Glioblastoma	Weak
IMR-32	Neuroblastoma	None
Colo 205	Colon	None
LOVO	Colon	None
SW403	Rectum	None
SW480	Colorectal adenocarcinoma	None
SW620	Colorectal adenocarcinoma	None

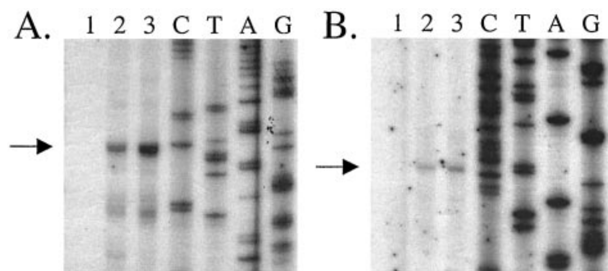

Fig. 5. Primer extension analysis of the *XAGE-1* gene. *A*, primer extension analysis of *XAGE-1a* performed using the primer Xagext.4 to define the 5′-most transcriptional start site. *Lane 1*, no RNA; *Lane 2*, testis RNA; *Lane 3*, Ewing's sarcoma cell line TC71 RNA. *B*, primer extension analysis of *XAGE-1b* performed using the primer Xagext.3 to define the position of the downstream start site. *Lane 1*, no RNA; *Lane 2*, testis RNA; *Lane 3*, Ewing's sarcoma cell line TC71 RNA. *Arrows*, the primer extension products. The DNA sequencing ladder is labeled with the complementary sequence CTAG.

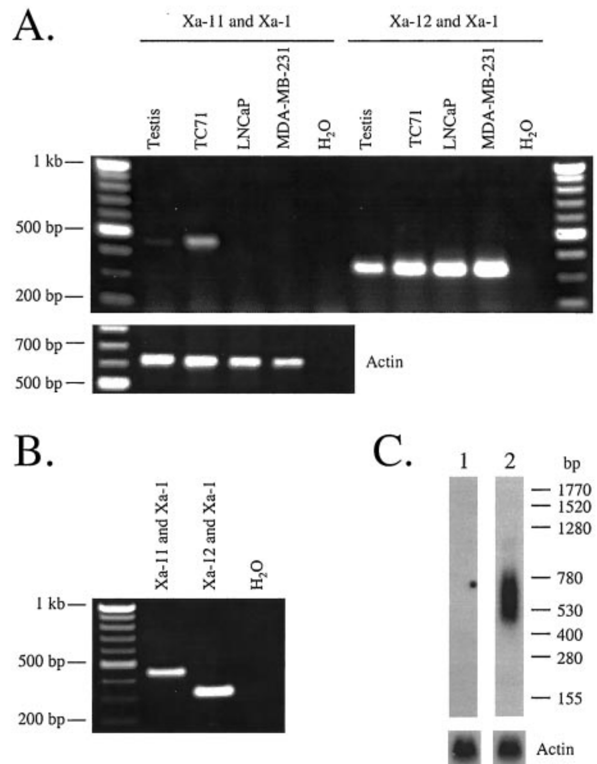

Fig. 6. Relative abundance of *XAGE-1* transcripts. *A*, RT-PCR analysis of *XAGE-1a* and *XAGE-1b* expression. The PCR reactions were performed using cDNAs derived from testis, TC71, LNCaP, and MDA-MB-231 with primers specific for *XAGE-1* (*top panel*), either Xa-11 and Xa-1, which generate a 453-bp product, or Xa-12 and Xa-1, which generate a 332-bp product (see Fig. 1*B*) or actin primers, Ori-Actin-F and Ori-Actin-R, producing a 640-bp product (*bottom panel*). Reactions performed without template are labeled H_2O. The PCR reactions were run on a 1.2% agarose gel and visualized by ethidium bromide staining. *B*, as a positive control, the pCRII-XAGE plasmid was used as a template for PCR amplification of *XAGE-1* using either primers Xa-11 and Xa-1 or Xa-12 and Xa-1. The PCR reactions were run on a 1.5% agarose gel and stained with ethidium bromide. *C*, Northern blot analysis of the *XAGE-1a* and *XAGE-1b* transcripts. Each lane contains poly(A) RNA (2 μg) from the TC71 cell line. *Lane 1*, membrane probed with a 130-bp DNA fragment unique to the *XAGE-1a* transcript (Fig. 1*B*, *Probe A*). *Lane 2*, membrane probed with a 130-bp probe common to both *XAGE-1a* and *XAGE-1b* transcripts (Fig. 1*B*, *Probe B*). The two membranes were stripped and analyzed with a β-actin probe to verify equal loading (*lower panel*). *On the right in nucleotides*, RNA size markers.

TC71 poly(A) RNA (Fig. 6*C*). A 130-bp DNA fragment unique to the 5′ region of *XAGE-1a* (Fig. 1*B*, *Probe A*) and a 130-bp DNA fragment in the common region of the two *XAGE-1* transcripts (Fig. 1*B*, *Probe B*) were used as probes. After several attempts with decreased stringency of hybridization and wash conditions, no hybridization signal was observed in the TC71 lane using probe A, which is unique to *XAGE-1a* (Fig. 6*C*, *Lane 1*). In contrast, probe B, which hybridizes to both *XAGE-1a* and *XAGE-1b*, reacted strongly with a region on the blot that spans from ~450 bp to 800 bp (Fig. 6*C*, *Lane 2*). This result indicated that several *XAGE-1b* transcripts exist, ranging in size from 450 to 800 bp. These could be attributable to different lengths of the poly(A) tails or to the use of an alternative poly(A) signal located farther downstream. The *XAGE-1* gene contains a second poly(A) signal positioned 202 bp downstream from the first AATAAA (Fig. 1*B*). To investigate whether the second poly(A) signal sequence is used, resulting in a longer transcript, Northern blot analysis was performed using a 130-bp DNA fragment located between the two poly(A) signal sequences as a probe (Fig. 1*B*, *Probe C*). No specific bands were detected, which suggested that the first poly(A) signal is most often used (data not shown). Although the *XAGE-1b* transcript is present in a range of sizes, transcripts initiating at the *XAGE-1b* start site (Fig. 1, *A* and *B*) constitute the most dominant form of *XAGE-1*.

Downloaded from http://aacrjournals.org/mct/article-pdf/1/7/441/2317761/gd0702000441.pdf by guest on 10 October 2024

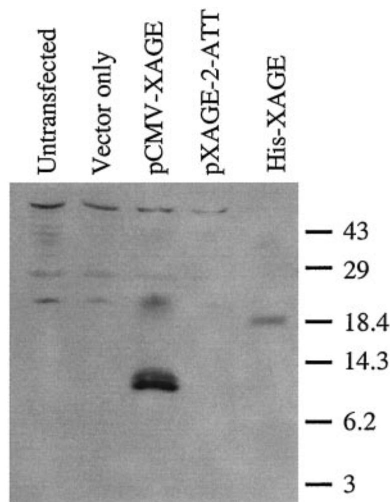

Fig. 7. *XAGE-1a* encodes a 9 kDa protein. Whole-cell lysates prepared from cells transfected with pcDNA3, pCMV-XAGE, or pXAGE-2-ATT were analyzed for the presence of the XAGE-1 protein by Western blot analysis. Samples (30 μg) were run on a Tris/Tricine 16.5% polyacrylamide gel and the membrane was probed with a polyclonal antibody against XAGE-1. As a positive control, 100 ng of the His-XAGE fusion protein was run on the gel. *On the right*, molecular mass standards in kDA.

Translation of *XAGE-1* Initiates with the Second In-Frame ATG. The *XAGE-1a* transcript contains two in-frame ATG translational start codons (Fig. 1, *A* and *B*). The entire ORF encodes a putative 16 kDA protein; however, translation from the second ATG would yield a protein with a molecular mass of 9 kDa. To determine which ATG translational start codon is most likely used, a transfection experiment was performed. A cDNA encoding the entire *XAGE-1* ORF and 21 bp 5′ of the first putative ATG start codon was cloned into pcDNA3 under the regulation of the CMV promoter. This plasmid is referred to as pCMV-XAGE. A human embryonic kidney cell line, 293T, was transfected with either pcDNA3 or pCMV-XAGE, and after 48 h, whole-cell lysates were generated. A Western immunoblot analysis was performed using polyclonal antiserum generated against a ΔPE-XAGE fusion protein (see "Materials and Methods"). As a positive control for the antiserum, a His-XAGE fusion protein, consisting of the full-length XAGE-1 protein with six histidine residues on the NH_2 terminus, was generated in *E. coli* and purified. The molecular weight of the recombinant His-XAGE fusion protein is ~17,300. A single reactive band was detected in the His-XAGE lane (Fig. 7). No specific reactive bands were observed in the lanes containing the untransfected or pcDNA3 cell lysates (Fig. 7). A 9 kDa reactive protein was observed when cells were transfected with pCMV-XAGE. Neither a 9 kDA nor a 16 kDa band was detected when the prebled antiserum was used (data not shown). To verify that translation of the 9 kDa protein was initiated from the second ATG in the ORF in pCMV-XAGE, site-directed mutagenesis was performed to change the second ATG codon to ATT, generating the plasmid pXAGE-2-ATT. This mutation resulted in the loss of the 9 kDa reactive protein (Fig. 7), which indicated that translation of the *XAGE-1a* transcript begins with the second ATG in the ORF.

Discussion

XAGE-1 is a member of the family of CT antigens that has a restricted expression pattern in normal tissues, with expression predominantly in the testis (1). Nevertheless, members of this family are expressed in a wide range of cancers (3, 4). The EST database predicts that *XAGE-1* is only expressed in testis, Ewing's sarcomas, and alveolar rhabdomyosarcomas (12); however, the coverage of the EST database is incomplete (22). Experimentally, we determined that *XAGE-1* is also expressed in other cancers of various histological types. *XAGE-1* is not only expressed in relatively rare bone and muscle cancers, it is also expressed in cancers that are much more common, such as breast, lung, and prostate cancers. We were especially interested in results obtained directly from *in situ* hybridization in fixed breast and prostate tissue sections of normal *versus* carcinomatous components (Fig. 4) because tissue sections yield direct visibility of target cells, an advantage over the inherent limitations of established tumor cell lines. The approach also confirmed higher signal intensity in the malignant tissues over benign elements. Our results reported here indicate that *XAGE-1* is weakly expressed in normal breast tissue but is abundantly expressed in many of the breast cancer samples tested (see Figs. 3 and 4). Of the breast cancer cell lines studied, *XAGE-1* was expressed only in ER-negative cell lines, whereas no expression was detected in ER-positive cell lines (Table 2). Determining whether the *XAGE-1* gene is regulated by estrogen and whether XAGE-1 could be a marker for cancer progression will require further investigation.

Similar to other members of the CT antigen family such as *MAGE*, *BAGE*, *GAGE*, and *NY-ESO-1* (3, 19, 20), *XAGE-1* is expressed in a significant number of lung squamous cell carcinomas, adenocarcinomas, SCLCs, and NSCLCs studied. In addition, like *MAGE* and *GAGE* (3), *XAGE*-1 is expressed in prostate cancer. *In situ* hybridization visibly demonstrates that *XAGE-1* is weakly expressed in the normal prostate epithelial cells but is abundantly expressed in the prostate cancer cells (Fig. 4, *C* and *D*). After a survey of numerous cancer cell lines (Table 2), *XAGE-1* revealed an expression pattern similar to other CT antigens, with the exception that it is expressed in a chronic myelogenous leukemia (CML-K562; Fig. 2), a T-cell lymphoma cell line, and a histiocytic lymphoma cell line (Table 2). Expression of CT antigens in leukemias and lymphomas is uncommon.

To further our characterization of the *XAGE-1* transcript, we performed a primer extension analysis of *XAGE-1*, which indicated that there are two distinct transcriptional start sites, both in exon 1 (Fig. 1*A*). The first, *XAGE-1a*, is located 8 bp upstream from the start site previously defined by RACE-PCR (13). This transcript contains two in-frame ATG translational start codons, which could encode either a 16 kDa or 9 kDa protein, respectively. The second, *XAGE-1b*, is located 58 bp downstream from the first potential ATG translational start codon located in *XAGE-1a*. The significance of the location of the *XAGE-1b* start site is that this transcript contains only the second in-frame ATG and therefore, could encode only the 9 kDa protein.

Because the two overlapping *XAGE-1* transcripts could potentially encode different XAGE-1 proteins, we wanted to

Downloaded from http://aacrjournals.org/mct/article-pdf/1/7/441/2317761/gd0702000441.pdf by guest on 10 October 2024

determine whether one transcript was more abundant than the other. RT-PCR analysis was performed using a 5′ PCR primer unique to *XAGE-1a* and a 3′ PCR primer common to both transcripts (Fig. 6*A*). Extremely weak bands or no products were observed with these primers, as compared with the abundant products present when 5′ and 3′ PCR primers common to both transcripts were used. This result implies that *XAGE-1a* is weakly expressed, compared with *XAGE-1b*. We further quantitated this finding by performing Northern blot analysis. Despite several attempts at using a DNA probe unique to *XAGE-1a* (Fig. 1*B*, *Probe A*) for Northern blot analysis, no reactive band was detected on the membrane (Fig. 6*C*, *Lane 1*). This suggests that expression of *XAGE-1a* is so weak that Northern blot analysis is not sensitive enough to detect it. Because only an extremely weak product was present using a 5′ PCR primer unique to *XAGE-1a* in RT-PCR analysis (Fig. 6*A*), it is not surprising that a reactive band was not observed using a probe unique to *XAGE-1a* in Northern blot analysis. In contrast, Northern blot analysis using a DNA fragment common to both *XAGE-1a* and *XAGE-1b* as a probe (Fig. 1*B*, *Probe B*) revealed a strong reactive region spanning from ∼450 bp to 800 bp (Fig. 6*C*, *Lane 2*).

One explanation for the different transcript lengths is the use of an alternative poly(A) signal farther downstream. Although *XAGE-1* does contain a second poly(A) signal, our analysis indicates that the first poly(A) signal sequence is used more often than the second one. Therefore, the calculated length of the *XAGE-1b* transcript, based on the use of the first poly(A) signal, is 466 bp excluding the poly(A) tail. The extensive range in transcript length observed by Northern blot analysis may be caused by the variation in size of the poly(A) tail. Nevertheless, we cannot rule out the possibility that Probe B (Fig. 1*B*), which is common to both *XAGE-1a* and *XAGE-1b*, is also hybridizing to the *XAGE-1a* transcript and that this contributes to the upper reactive bands (Fig. 6*C*, *Lane 2*). However, both the Northern blot analysis using the probe unique to *XAGE-1a* and the RT-PCR analysis suggest that *XAGE-1b* is much more abundant than *XAGE-1a*. Taken together, these data indicate that *XAGE-1a* is weakly expressed, whereas *XAGE-1b* seems to be the dominant transcript. Definition and characterization of the two *XAGE-1* promoters will require further investigation. It has been shown previously that the *MAGE* promoter is activated by demethylation, which is a consequence of genome-wide demethylation in the tumor cells (23). Whether *XAGE-1* is activated by demethylation still needs to be determined.

Although the *XAGE-1a* transcript contains two in-frame ATG translational start codons, the *in vitro* transfection analysis revealed that translation initiates at the second ATG and, consequently, a 9 kDa protein is made (Figs. 1*B* and 7). The Kozak sequence for the second ATG translation initiation site is more conserved than the first (24), which is consistent with translation initiating at the second ATG. In addition, the 9 kDa protein is encoded by the most abundant transcript, *XAGE-1b*. Although these *in vitro* results suggest that XAGE-1 is a 9 kDa protein, we cannot eliminate the possibility that the native protein is 16 kDa or that both the 16 kDa and 9 kDa proteins are made by cells. Because the 9 kDa protein is missing the hydrophobic region in the NH_2 terminus, this protein is probably not a membrane protein.

To determine the size of the native protein as well as the cellular location, we performed Western immunoblot analysis on nuclear, cytoplasmic, and membrane fractions from TC71 and U2OS, Ewing's sarcoma, and osteosarcoma cell lines, respectively, which had all been previously shown to express *XAGE-1* (13). Unfortunately, we were not able to detect a specific band in the fractionated cell extracts (data not shown). The detection limit for the XAGE-1 antiserum generated against the ΔPE-XAGE fusion protein is 10 ng, as determined by Western immunoblot analysis against known amounts of the His-XAGE fusion protein (data not shown). The XAGE-1 protein may be present in the cells at concentrations too low to be detected by this antiserum. To investigate the possibility that XAGE-1 is degraded too rapidly to be detected, we treated TC71 cells for 12 h with 10 μM lactacystin, a specific and irreversible inhibitor of the ubiquitin-proteasome pathway (25, 26), to prevent the possible degradation of XAGE-1. Western immunoblot analysis was performed on nuclear, cytoplasmic, and membrane fractions generated from TC71 cells treated with either lactacystin or vehicle alone. No specific bands were detected in either case (data not shown). It appears that the antiserum generated against the ΔPE-XAGE fusion protein is not sensitive enough to detect the native protein in cells. Therefore, we are in the process of developing a better antibody to the XAGE-1 protein.

In this report, we have shown that *XAGE-1* RNA is detected in a wide range of cancers including lung, breast, and prostate. Because the *XAGE-1* gene is expressed in a large proportion of tumors of various histological types and has the characteristics of a cytosolic protein, XAGE-1 may constitute a useful target for vaccine-based therapies of cancer.

Acknowledgments

We thank Drs. Paul Egland, Tapan Bera, Satoshi Nagata, and Rangan Maitra for helpful discussions and critical reading of the manuscript. We thank Verity Fogg for excellent tissue-culture support and Anna Mazzuca for exceptional editorial assistance.

References

1. Takahashi, K., Shichijo, S., Noguchi, M., Hirohata, M., and Itoh, K. Identification of MAGE-1 and MAGE-4 proteins in spermatogonia and primary spermatocytes of testis. Cancer Res., *55:* 3478–3482, 1995.

2. Boon, T., Coulie, P. G., and Van den Eynde. B. Tumor antigens recognized by T cells. Immunol. Today, *18:* 267–268, 1997.

3. De Smet, C., Lurquin, C., De Plaen, E., Brasseur, F., Zarour, H., De Backer, O., Coulie, P. G., and Boon, T. Genes coding for melanoma antigens recognised by cytolytic T lymphocytes. Eye, *11:* 243–248, 1997.

4. Old, L. J., and Chen, Y-T. New paths in human cancer serology. J. Exp. Med., *187:* 1163–1167, 1998.

5. van der Bruggen, P., Traversari, C., Chomez, P., Lurquin, C., Deplaen, E., Van den Eynde, B., Knuth, A., and Boon, T. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science, *254:* 1643–1647, 1991.

6. Boel, P., Wildmann, C., Sensi, M. L., Brasseur, R., Renauld, J., Coulie, P., Boon, T., and van der Bruggen, P. *BAGE*: a new gene encoding an antigen recognized by cytolytic T lymphocytes. Immunity, *2:* 167–175, 1995.

Downloaded from http://aacrjournals.org/mct/article-pdf/1/7/441/2317761/gd0702000441.pdf by guest on 10 October 2024

7. Van den Eynde, B., Peeters, O., De Backer, O., Gaugler, B., Lucas, S., and Boon, T. A new family of genes coding for an antigen recognized by autologous cytolytic T lymphocytes on a human melanoma. J. Exp. Med., *182:* 689–698, 1995.

8. Gure, A. O., Tureci, O., Sahin, U., Tsang, S., Scanlan, M. J., Jager, E., Knuth, A., Pfeundschuh, M., Old, L. J., and Chen, Y-T. SSX, a multigene family with several members transcribed in normal testis and human cancer. Int. J. Cancer, *72:* 965–971, 1997.

9. Chen, Y-T., Boyer, A. D., Viars, C. S., Tsang, S., Old, L. J., and Arden, K. C. Genomic cloning and localization of CTAG, a gene encoding an autoimmunogenic cancer-testis antigen NY-ESO-1, to human chromosome Xq28. Cytogenet. Cell Genet., *79:* 237–240, 1997.

10. Tureci, O., Sahin, U., Zwick, C., Koslowski, M., Seitz, G., and Pfeundschuh, M. Identification of a meiosis-specific protein as a new member of the class of cancer/testis antigens. Proc. Natl. Acad. Sci. USA, *95:* 5211–5216, 1998.

11. Vasmatzis, G., Essand, M., Brinkmann, U., Lee, B., and Pastan, I. Discovery of three genes specifically expressed in human prostate by expressed sequence tag database analysis. Proc. Natl. Acad. Sci. USA, *95:* 300–304, 1998.

12. Brinkmann, U., Vasmatzis, G., Lee, B., and Pastan, I. Novel genes in the PAGE and GAGE family of tumor antigens found by homology walking in the dbEST database. Cancer Res., *59:* 1445–1448, 1999.

13. Liu, X. F., Helman, L. J., Yeung, C., Bera, T. K., Lee, B., and Pastan, I. *XAGE-1*, a new gene that is frequently expressed in Ewing's sarcoma. Cancer Res., *60:* 4752–4755, 2000.

14. Wolfgang, C. D., Essand, M., Vincent, J. J., Lee, B., and Pastan, I. TARP: a nuclear protein expressed in prostate and breast cancer cells derived from an alternate reading frame of the T cell receptor γ chain locus. Proc. Natl. Acad. Sci. USA, *97:* 9437–9442, 2000.

15. Olsson, P., Bera, T. K., Essand, M., Kumar, V., Duray, P., Vincent, J., Lee, B., and Pastan, I. *GDEP*, a new gene differentially expressed in normal prostate and prostate cancer. Prostate, *48:* 231–241, 2001.

16. Kumar, V., and Collins, F. H. A technique for nucleic acid *in situ* hybridization to polytene chromosomes of mosquitoes in the Anopheles gambiae complex. Insect Mol. Biol., *3:* 41–47, 1994.

17. Bruggemann, E. P., Chaudhary, V., Gottesman, M. M., and Pastan, I. Pseudomonas exotoxin fusion proteins are potent immunogens for raising antibodies against P-glycoprotein. Biotechniques, *10:* 202–209, 1991.

18. Pear, W. S., Nolan, G. P., Scott, M. L., and Baltimore, D. Production of high-titer helper-free retroviruses by transient transfection. Proc. Natl. Acad. Sci. USA, *90:* 8392–8396, 1993.

19. Van den Eynde, B. J., and van der Bruggen, P. T cell defined tumor antigens. Curr. Opin. Immunol., *9:* 684–693, 1997.

20. Lee, L., Wang, R. F., Wang, X., Mixon, A., Johnson, B. E., Rosenberg, S. A., and Schrump, D. S. NY-ESO-1 may be a potential target for lung cancer immunotherapy. Cancer J. Sci. Am., *5:* 20–25, 1999.

21. Chen, Y. T., and Old, L. J. Cancer-testis antigens: targets for cancer immunotherapy. Cancer J. Sci. Am., *5:* 16–17, 1999.

22. Zhang, L., Zhou, W., Velculescu, V. E., Kern, S. E., Hruban, R. H., Hamilton, S. R., Vogelstein, B., and Kinzler, K. W. Gene expression profiles in normal and cancer cells. Science, *276:* 1268–1272, 1997.

23. De Smet, C., De Backer, O., Faraoni, I., Lurquin, C., Brasseur, F., and Boon, T. The activation of human gene *MAGE-1* in tumor cells is correlated with genome-wide demethylation. Proc. Natl. Acad. Sci. USA, *93:* 7149–7153, 1996.

24. Kozak, M. Initiation of translation in prokaryotes and eukaryotes. Gene, *234:* 187–208, 1999.

25. Bush, K. T., Goldberg, A. L., and Nigam, S. K. Proteasome inhibition leads to a heat-shock response, induction of endoplasmic reticulum chaperones, and thermotolerance. J. Biol. Chem., *272:* 9086–9092, 1997.

26. Fenteany, G., Standaert, R. F., Lane, W. S., Choi, S., Corey, E. J., and Schreiber, S. L. Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. Science, *268:* 726–731, 1995.

Downloaded from http://aacrjournals.org/mct/article-pdf/1/7/441/2317761/gd0702000441.pdf by guest on 10 October 2024