Vol. 202, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1994 Pages 429-436

TRANSCRIPTIONAL REGULATION OF CATHEPSIN B EXPRESSION IN B16 MELANOMAS OF VARYING METASTATIC POTENTIAL

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Received May 27, 1994

Summary: The highly metastatic B16a melanoma has been shown to express higher levels of cathepsin B (CB) mRNA when compared to the less metastatic variants, B16-F1 and B16-F10, and with normal mouse tissues. This increased expression is now shown to be due to increased gene transcription by nuclear run-off assays and measurements of mRNA stability. Transient expression assays, using promoter fragments from the mouse and human CB genes, demonstrated that both promoters were more active in B16a than in the less metastatic melanomas, B16-F1 and B16-F10. The differential gene expression did not depend on the presence of multiple Sp1 sites in both promoters. A Gel shift assay revealed a specific CB promoter binding protein whose levels are correlated with CB expression and the metastatic potential of the three B16 melanoma variants. These results indicate that the increased expression of CB in the B16a melanoma is due to a specific increase in the amount or activity of a transcriptional activator of the CB gene. The ability of the human CB promoter to activate gene expression in B16a melanoma cells suggests similarities in the regulation of CB expression in tumors from humans and mice.

The increased expression, secretion, and aberrant subcellular distribution of cathepsin B (CB) have been linked to cellular transformation and metastasis in tumors from rodents and humans (1-9). A comparison of mRNA levels for five lysosomal proteinases in three murine melanoma variants, B16-F1, B16-F10, and B16a, revealed that only CB was elevated in the metastatic variants (8). In addition, all three melanoma variants were found to contain three transcripts for CB, 2.2, 4.0, and 5.0 kb, while only the 2.2 kb message was seen in

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Abbreviations: CB, cathepsin B; CAT, chloramphenicol acetyltransferase; Glut-1; glucose transporter-1; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; SDS, sodium dodecyl sulfate.

0006-291X/94 $5.00

the normal mouse tissues studied (8,10). A comparison of these mRNA isoforms with the CB gene demonstrated that they resulted from the use of alternative polyadenylation signals during the processing of the 3' untranslated end of the CB pre-mRNA (10,11). CB mRNA isoforms which differ in their 5'untranslated ends have also been reported in Kirsten virus-transformed BALB/3T3 cells (12). These may have arisen from the presence of multiple promoters and transcription start sites in the murine CB gene (12). To explore the relationship between CB expression and tumor metastasis we have examined the stability of the CB messages and the transcriptional activity of the CB gene. We have also measured the transcriptional activity of a DNA fragment derived from a putative CB gene promoter and have shown that it is capable conferring the differential gene expression observed among the three B16 melanoma variants.

MATERIALS AND METHODS

Cell culture. *The murine melanoma variants, B16-F1, B16-F10, and B16a, their propagation* in culture, and their capacity to colonize the lung following intravenous and subcutaneous injection, have been described previously (8,13).

cDNA probes and expression vectors. Mouse Glut-1 cDNA and *gamma*-actin cDNA were gifts from Drs. S. Nagamatsu and P. Engler, respectively (The University of Chicago, Chicago, IL). The mouse CB cDNA has been described (14). The plasmids pB250 and pE250 contain a 240 bp fragment from the mouse CB gene (-155 to +85) (11) cloned into the reporter vectors pCAT-Basic and pCAT-Enhancer (Promega, Madison, WI). Plasmids pB500 and pE500 contain a corresponding 478 bp fragment (-341 to +137) from the human CB gene (15). The plasmid pACT-gal, which contains ß-galactosidase fused to the actin gene promoter, was a gift from Dr. Nobuya Inagaki (Kyoto University School of Medicine, Kyoto, Japan).

Measurement of mRNA stability. Actinomycin D was added at a final concentration of 5 μg/ml to near confluent monolayers of B16 melanoma cells in complete media in T-75 flasks. At various intervals from 0 to 12 h, total RNA was extracted with guanidinium thiocyanate from four flasks and purified by centrifugation through a $CsCl_2$ cushion as described previously (8). For Northern blot analyses, 15 μg of total RNA/lane were fractionated by electrophoresis on 1.2% agarose — 2.2 M formaldehyde gels and transferred onto nitrocellulose filters. For dot blot analyses, varying amounts (0.5-5 μg) of denatured RNA were spotted directly on nitrocellulose filters (16). The filters were probed with ^{32}P-labeled CB and Glut-1 cDNAs (8) and hybridization signal intensities were measured by densitometry (LKB Ultrascan Laser Densitometer) after autoradiography.

Nuclear Runoff Transcription. The nuclear run-off transcription assay was performed as described by Greenberg and Bender (17) with modification. Nuclei were prepared from the three B16 melanoma variants by lysis in isotonic buffer, pH 7.4, containing 0.1% Nonidet P-40, with 10 gentle strokes of a Dounce homogenizer fitted with a type B pestle. The nuclei were then sedimented at 500 X g at 4°C, suspended in glycerol storage buffer (17), and frozen at -70°C. The reaction was initiated by adding 10^7 thawed nuclei in 200 μl storage buffer to an equal volume of 2 X reaction buffer (17) containing 120 units of RNasin, 1 mM each of ATP, GTP and CTP, and 100 μCi [α-^{32}P]UTP. After a 30 min incubation at 30°C, the reaction was terminated by the addition of guanidinium thiocyanate. The RNA products were isolated (8) and dissolved in 10 mM HEPES, pH 7.3, 10 mM EDTA, pH 7.5, 0.2% SDS. Plasmids containing mouse CB cDNA, mouse *gamma*-actin cDNA and the vector pGEM-4Z (Promega) were linearized with appropriate restriction enzymes, denatured, and applied to nitrocellulose filters (10 μg/well). Equal amounts (~10^6 CPM) of radiolabeled RNA product from each cell line were added to 500 μl of 10 mM HEPES, pH 7.3, 10 mM EDTA, pH 7.5, 0.2% SDS, 0.6 M NaCl, and incubated with each dot blot filter for 48 hours at 65°C. After washing the filters (17) the hybridization products were visualized by autoradiography.

Transient CAT Expression. On the day before transfection 10^6 cells were plated on 100 mm tissue culture dishes. Two h before the addition of DNA the cells were replenished with fresh medium. Ten μg of the appropriate CAT construct and 10 μg of pACT-gal were co-transfected into cells using the Lipofectin reagent (Gibco BRL, Grand Island, NY) according to the instructions of the manufacturer. Cells were harvested after 48 h and CAT assays were performed after normalization for ß-galactosidase activity (18).

Gel-Mobility Shift Assay. A 240 bp fragment containing the CB promoter (-155 to +85) was labeled with [α-^{32}P]dCTP and T4 DNA polymerase (Boehringer-Mannheim) (19). DNA binding was carried out at room temperature for 30 min in a 20 μl reaction volume containing 2 μg poly(dI-dC)-poly(dI-dC) (Pharmacia), 10 μg nuclear extract protein (20), and the radioactive genomic fragment (20,000 CPM, .0.01 pmol). For competition experiments, 0.25 pmol of the unlabeled 240 bp promoter fragment, or 0.25 pmol PGEM-4Z DNA, or 3.5 pmol of Sp1 oligonucleotides (5'-ATTCGATCGGGGCGGGGCGAGC-3', 3'-TAAGCTAGCCCCGCCCCGCTCG-5' were added 10 min prior to the addition of the radiolabeled promoter fragment. The binding reaction mixtures were loaded onto a pre-electrophoresed 4% polyacrylamide gel and electrophoresis was carried out at 30 mA in 40 mM Tris·acetic acid, 20 mM EDTA for 4 h.

RESULTS

Stability of Cathepsin B mRNA in B16 Melanoma Variants. The stability of CB mRNA in the mouse melanoma variants B16-F1 and B16a was determined by northern (Fig. 1) and dot blot hybridization after the addition of the RNA polymerase II inhibitor, actinomycin D, to the cells. Mouse Glut-1 mRNA, which is reported to have a half-life of 1 to 5 h depending on conditions (21,22), was measured as a positive control. Logarithmic plots of the densitometric data from the dot blots are presented in Fig. 2. Half-lives were estimated to be about 4 h for Glut-1 mRNA in both B16-F1 and B16a melanoma cells in culture. In contrast, CB mRNA is very stable in both melanomas under these same conditions, with little evidence of degradation observed after 9 and 12 h. Extrapolation of the data points shown in Fig. 2 yielded an estimated half-life of about 35 h for the CB messages in both

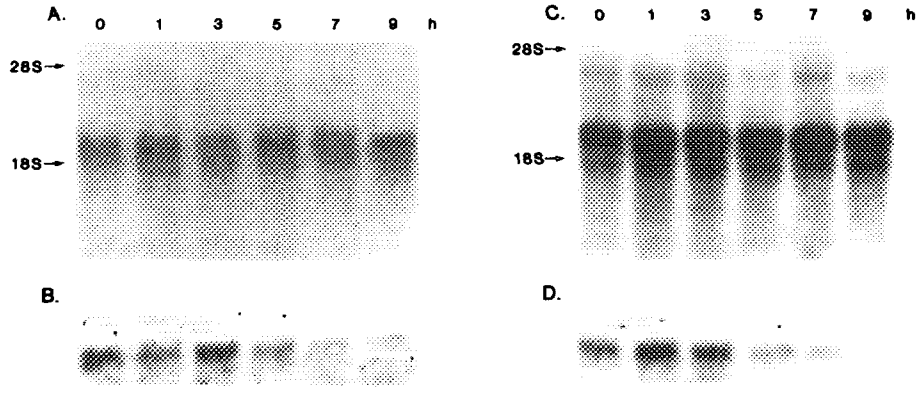
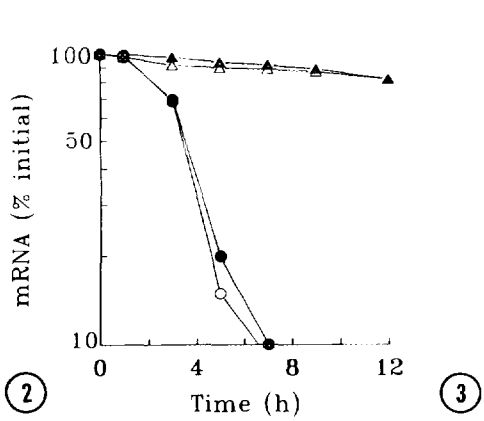


Fig. 1. Northern blot analysis of CB and glut-1 mRNA stabilities in cultured mouse B16-F1 and B16a melanoma cells after treatment with 5 μg/ml actinomycin D. Total RNA was isolated from B16-F1 (panels A and B) and B16a (panels C and D) cells at 2 h intervals after addition of actinomycin D to the culture medium. Following fractionation on 1.2 % agarose-2.2M formaldehyde gels and transfer onto nitrocellulose filters, blots were incubated with ^{32}P-labeled mouse CB cDNA (A and C). The same blots were stripped of the CB probe and reprobed with ^{32}P-labeled mouse Glut-1 cDNA (B and D).



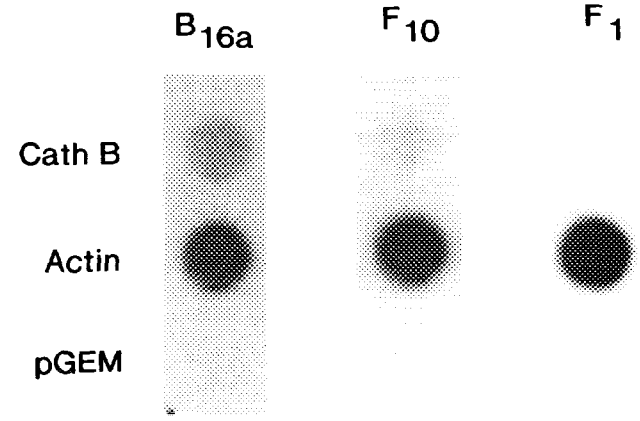


Fig. 2. First order decay of CB and Glut-1 mRNA in B16-F1 and B16a melanoma cells. Autoradiographs of RNA dot blots (data not shown) were quantified by densitometry. Relative density readings were plotted as a function of the time after transcription inhibition by actinomycin D. Each time point is the average of four separate experiments. Triangles, CB mRNA; circles, Glut-1 mRNA; solid symbols, B16a cells; open symbols, B16-F1 cells.

Fig. 3. Runoff transcription in nuclei from B16 melanoma variants. ^{32}P-labeled nuclear RNAs from B16-F1, B16-F10 and B16a melanoma cells were hybridized to mouse CB cDNA, mouse *gamma*-actin cDNA and the plasmid pGEM-4Z (Promega Corp., Madison, WI) as described in "Materials and Methods".

cells. Thus, the much higher levels of CB mRNA seen in B16a cells as compared with B16-F1 (Fig. 1 and Ref. 8) cannot be due to differences in mRNA stability.

Rates of Cathepsin B Transcription Initiation in B16 Melanoma Variants. Run-off transcription assays were performed to determine relative rates of CB gene transcription in nuclei isolated from the three variant melanoma cell lines. The hybridization signals shown in Fig. 3 reflect the steady state levels of transcription initiation of CB pre-mRNA. This signal was approximately 6- and 4-fold more intense with nuclei from B16a cells than from B16-F1 and B16-F10 cells, respectively, after normalization for loading using the actin signal. This result correlates with the relative abundances of CB messages seen in these same cells (8). The absence of any hybridization to the immobilized plasmid pGEM-4Z DNA in Fig. 3 demonstrated the specificity of the signal.

Activity of a Putative Cathepsin B Promoter in B16 Melanoma Variants. Transient transfection/CAT expression assays were used to measure the ability of putative CB gene promoters to drive differential gene expression in the B16 melanoma variants. Transfections of the variant B16 melanoma cell lines with the different CB promoter—CAT gene constructs were repeated three times with identical results. In all transfections the control plasmid, pACT-gal, gave ß-galactosidase activities which varied by less than 7%, indicating that any variations in CAT expression among the melanoma variants could not be due to differences in transfection efficiency. A representative example in Fig. 4 shows that a fragment of the CB gene (-155 to +85), that we previously identified as a putative

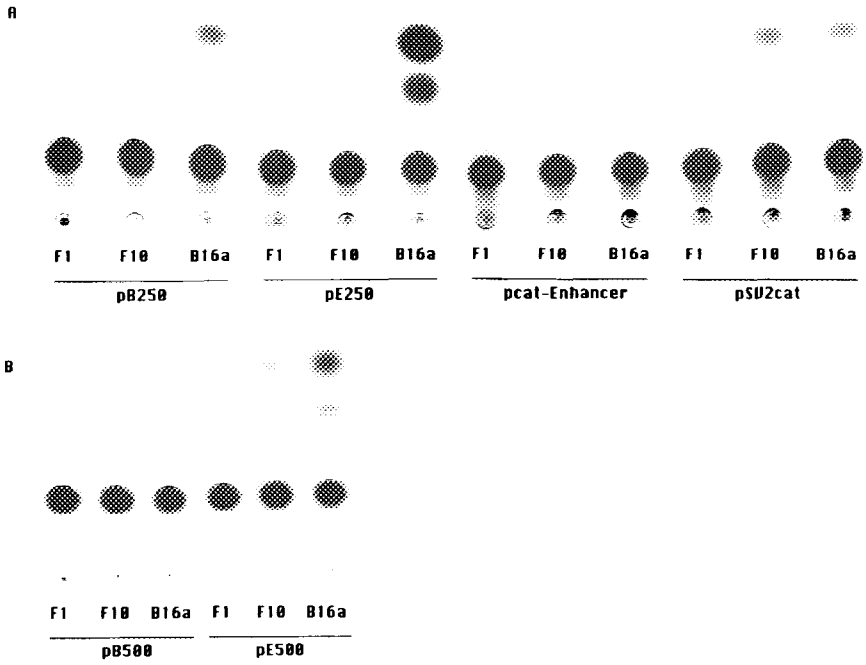


Fig. 4. Transactivation of the CB promoter in B16-F1, B16-F10 and B16a cells. CAT activity was measured as described in "Materials and Methods". Panel A, pB250 contains the mouse CB promoter (-155 to +85); pE250 contains both the mouse CB promoter and SV40 enhancer; pCAT-Enhancer contains the SV40 enhancer; and pSV_2cat contains both SV40 promoter and enhancer sequences. Panel B, pB500 contains the human CB promoter (-341 to +137); and pE500 contains both the human CB promoter and SV40 enhancer.

CB gene promoter (11), is indeed capable of directing the full range of expression seen for the CB gene in the three melanoma variants. Thus, substantially higher CAT expression was detected in transfected B16a cells than in either B16-F1 or B16-F10. This was seen with both pB250, which contains only the putative mouse CB promoter, and pE250 which contains in addition an SV40 enhancer. A similar region (-341 to +137) from the putative human CB promoter (15) in plasmids pB500 and pE500 produced a similar differential expression of CAT within the three murine melanomas (Fig. 4). In both cases, however, the human promoter was substantially less active than the mouse promoter. In control experiments the vector pCAT-Enhancer, which contains an SV40 enhancer but no promoter, produced no CAT activity. Transfectants harboring pSV_2CAT (containing both SV40 enhancer and promoter) produced similar levels of CAT activity among the three melanoma variants and gave weaker signals than pE250 or pE500. However, pSV_2CAT yielded high levels of CAT activity when transfected into COS 7 cells (data not shown), excluding the possibility of a defective SV40 promoter in this plasmid. Thus, the CB promoter is more active than the SV40 promoter in the B16a melanoma.

Detection of Cathepsin B-promoter Binding Protein in B16a Cells. The radiolabeled 240 bp *PstI* fragment (-155 to +85), shown above to have promoter functions, was used to

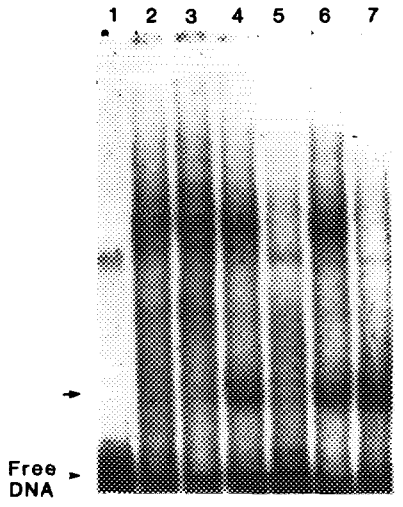


Fig. 5. Gel-retardation analysis of nuclear binding proteins from B16 melanoma variants. A ^{32}P-labeled 240 bp *Pst*I restriction fragment from mouse CB gene (-155 to +85) was incubated in the absence (lane 1) or presence of nuclear extracts from B16-F1 (lane 2) or B16-F10 (lane 3) or B16a (lane 4) melanoma variants. Some incubations included a 25-fold molar excess of the non-radioactive 240 bp fragment (lane 5), a 350-fold molar excess of Sp1-specific oligonucleotide sequences (lane 6), or a 25-fold molar excess of pGEM-4Z DNA (lane 7) which were added to a B16a nuclear extract 10 minutes prior to the addition of the radioactive promoter. The carrot indicates the position of the unretarded DNA fragment. The arrow indicates a specific DNA binding protein which is expressed in higher levels in B16a than in B16-F1 or B16-F10 cells.

detect nuclear DNA binding proteins in an electrophoretic mobility shift assay (Fig. 5). In lane 1, the labeled promoter fragment alone shows a single major band due to free DNA (indicated by a carrot) and traces of a minor constituent. Two major protein:DNA complexes were formed after addition of nuclear extracts from the three melanoma variants (lanes 2, 3 and 4). The amount of the slower-migrating complex was similar in extracts from all three variants, while the faster migrating complex (identified with an arrow) was clearly more abundant in B16a. Both complexes were competed by a 25-fold molar excess of the non-radioactive 240 bp promoter fragment (lane 5). However, only the slower-migrating band was competed by a 25-fold molar excess of pGEM-4Z DNA (lane 7), suggesting that it may be due to a nonspecific DNA binding protein. A 350-fold molar excess of Sp1 oligonucleotides failed to compete for formation of either complex (lane 6), suggesting that these CB promoter binding proteins were unlikely to be Sp1.

DISCUSSION

Recent results suggest the presence of multiple promoters in the CB gene from mice (12). We have previously identified and cloned the region of the CB gene corresponding to the 5'-untranslated end of CB mRNAs in murine B16a melanoma cells (10,11). We now show that a 240 bp 5'-*Pst*I fragment incorporating this region of the CB gene (-155 to +85) (11) does indeed have promoter activity when subcloned into a promoterless CAT expression vector. More importantly, we have demonstrated that this fragment can confer

a range of CAT expression in B16 melanoma variants which is similar to that seen for CB and which correlates with the metastatic potential of these cells. Furthermore, gel retardation binding assays employing this DNA fragment detected the highest levels of a corresponding DNA binding protein in the melanoma variant which expressed the highest level of CB mRNA. Thus, we have identified a CB gene promoter and a corresponding DNA binding protein, which may be responsible for the high levels of CB gene transcription associated with a metastatic phenotype in murine melanomas. We have also found that a fragment from the 5'-end of the human CB gene behaves in a similar manner in these CAT expression assays. This further suggests the important possibility that the DNA regulatory sequences and their cognate binding proteins which are responsible for the increased expression of CB in some metastatic tumors may be similar in humans and rodents.

A comparison of the human CB promoter with that of the murine promoter active in B16 melanomas reveals that they share the presence of extended GC-rich sequences which include putative binding sites for the transcription factor Sp1 (11,15). However, two lines of evidence suggest that the enhanced transcription of CB in the B16a melanoma cell is not entirely mediated by Sp1. First, a 350 fold molar excess of Sp1 specific oligonucleotides could not displace the promoter from the melanoma CB transcription factor in a DNA binding assay. Second, all three melanoma variants expressed similar low levels of CAT activity after transfection with the plasmid pSV_2-CAT, which contains 6 Sp1 binding sites (23). The SV40 early promoter and enhancer in pSV_2-CAT also contain regulatory sequences which bind other general and cell specific transcription factors, including AP-1, AP-2, and the octamer motif (23-26), so that these are also unlikely to be responsible for differences in CB expression in B16 melanoma variants.

In conclusion, we have demonstrated that the increased expression of CB in a highly metastatic B16a melanoma cell variant is due to increased gene transcription. We have shown that a putative mouse CB promoter, identified from analyses of the 5' ends of mouse CB mRNAs from kidney and B16 melanoma cells, is active in promoter assays. Furthermore, this promoter is capable of reproducing the differential CB gene expression observed among the B16 melanoma variants. We have also found in B16 cells a CB promoter binding protein whose levels parallel those of CB gene expression and the metastatic potential of the melanoma variants. The identification of this possible transcription factor should be important to the understanding of the up regulation of genes like CB and the generation of a metastatic phenotype in tumors.

ACKNOWLEDGMENTS

This work was supported in part by the Howard Hughes Medical Institute, The University of Chicago and NIH Grant DK13914, and by Loyola University of Chicago and Potts Estate Grant 842-04. We also wish to thank Raymond Carroll for assistance in data analysis and Richard M. Schultz for his critical reading of this manuscript.

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