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Ets-1 activates parathyroid hormone-related protein gene expression in tumorigenic breast epithelial cells

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Abstract

Parathyroid hormone-related protein (PTHrP) is produced by many tumors not associated with humoral hypercalcemia, including breast cancers. In this study, we used three human immortalized mammary epithelial cell lines that differ in tumorigenicity and PTHrP expression. Using RT-PCR we investigated 5′ and 3′ alternative splicing of PTHrP transcripts and promoter usage in the lines. Increased levels of P3-derived transcripts and the 1–139 mRNA isoform were observed in the most tumorigenic cell line. Transient transfection experiments identified elements close to P3 promoter that appeared to account for a portion of differential PTHrP expression among the three cell lines. Using site-directed mutagenesis, a previously described Ets-1/Sp1 binding site upstream of P3 was determined to be crucial for full activity of this promoter. RT-PCR and western blot evaluation of Ets family member expression found that Ese-1 was present in all three lines, but that appreciable levels of Ets-1 protein were present exclusively in the most tumorigenic line. Cotransfection of Ets-1 expression vectors activated PTHrP reporter constructs in the most tumorigenic line but not in the other cell lines. These findings suggest a potential mechanism by which PTHrP transcription may be regulated as a consequence of events that promote tumorigenic behavior in breast epithelial cells.

Keywords: PTHrP; Transcription; Ets factors; Promoter; Breast cancer

1. Introduction

Parathyroid hormone-related peptide (PTHrP) was initially discovered as a tumor-derived product associated with humoral hypercalcemia of malignancy (HHM). Eight of thirteen amino acids in the N-terminal portion of PTHrP are identical to PTH, and this homology accounts for the peptide's ability to bind to and stimulate the classical PTH/PTHrP receptor (Broadus and Stewart, 1994). Recent studies using targeted overexpression or disruption of the PTHrP gene have demonstrated that the peptide regulates developmental processes in the cartilaginous growth plate, skin, tooth and mammary gland (Wysolmerski et al., 1994, 1998; Karaplis et al., 1994; Foley et al., 1998). In contrast to the limited expression of PTH, the PTHrP gene is expressed at low levels by virtually all tissues during development or in the adult (Broadus and Stewart, 1994). Understanding the mechanisms that underlie PTHrP gene expression may provide further insight into the functional role of this peptide in both development and neoplastic disease.

The human PTHrP gene is a complex transcriptional unit composed of nine exons spanning more than 15 kb of genomic DNA (Broadus and Stewart, 1994) (Fig. 1). Transcription of the gene may initiate at any one of three promoters: P1 and P3 are canonical TATA promoters and initiate transcription at exons 1A and 2, respectively, while P2 is a high GC-content element that initiates transcription 11 nucleotides 5′ to the splice acceptor site of exon 1C (Vasavada et al., 1993).

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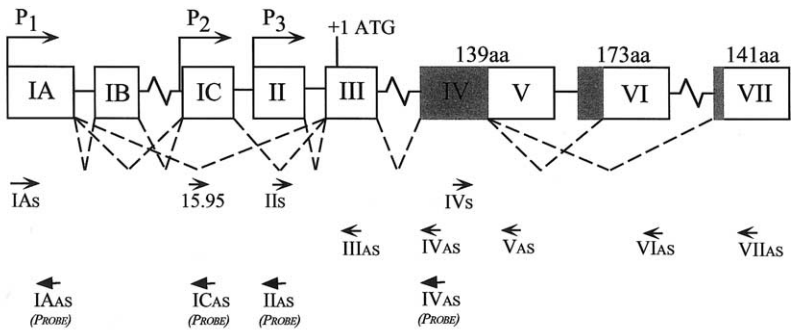

PCR Primer Combination	Transcripts Detected	Exons	Size of Product (bp)	Probe
II S + III AS	P3 Transcripts	II – III	273	II AS
15.95* + III AS	P2 Transcripts	IC – III	327	IC AS
IA S +III AS	P1 Transcripts	IA–IB–IC–III	610	IA AS
		IA–IC–III	517	IA AS
		IA–III	277	IA AS
IV S + IV AS	All Transcripts	IV	289	IV AS
IV S + V AS	139 a.a. isoform	IV – V	476	IV AS
IV S + VI AS	173 a.a. isoform	IV – VI	426	IV AS
IV S + VII AS	141 a.a. isoform	IV – VII	389	IV AS

Fig. 1. PTHrP gene and primers used in RT-PCR. On the top of the panel is a schematic representation of the genomic organization of the PTHrP gene where boxes indicate exons; shaded areas, sequences in the mature peptide; dotted lines, splicing patterns; arrows, promoter and transcription initiation sites; and +1, the translation start site (numbering of schematics and constructs used in this and subsequent figures is based upon the translation start site being +1). The arrows below the schematic indicate the relative positions of the primers and probes used in the semiquantitative RT-PCR analysis of PTHrP gene expression, 3′ splicing and promoter usage. * (Southby et al., 1995).

Alternative splicing of the 5′ noncoding exons (1A, 1B, 1C and 2) gives rise to multiple mRNA products, and only two exons are present in all PTHrP transcripts: exon III that encodes the prepro region, and exon IV which encodes the majority of the mature protein (Yasuda et al., 1989) (Fig. 1). There is also alternative splicing of 3′ exons that produces mRNAs which will be translated into PTHrP proteins of 139, 173, and 141 amino acids depending on whether exon IV is spliced to exon V, VI or VII, respectively (Mangin et al., 1988, 1990; Thiede and Rodan, 1988; Suva et al., 1989). In most cell lines, tumors and tissues studied to date the mRNA's that generate all three protein isoforms are frequently observed, but in certain cancer cell lines the 1–139 message appears to be the predominant form (Gillespie and Martin, 1994). The complexity of the PTHrP gene suggests that regulation may occur by transcriptional and post-transcriptional mechanisms, providing considerable opportunity for dysregulation in neoplastic diseases.

PTHrP production is not limited to those neoplasms associated with HHM, but is produced by a variety of human cancer cell lines and tumors, including breast cancer. For the most part, differential PTHrP expression among tumors of a specific cell type appears to result from altered transcription (Gillespie and Martin, 1994). In a limited number of cases, the *cis*-acting sequences that control PTHrP gene expression in specific tumors have been identified. In renal carcinoma cell lines, transcription from the P2 promoter appeared to be silenced by methylation of CpG dinucleotides in intron 2 (Holt et al., 1993). The high expression from the human P3 promoter or the rodent equivalent of this promoter has been reported to be dependent on Ets-1/Sp-1 sequences 20–40 bases upstream of the TATA box (Dittmer et al., 1993, 1994; Karperien et al., 1997; Foley et al., 1999).

In a previous study, we have reported that the PTHrP 1–139 mRNA was significantly increased in primary tumors from breast cancer patients who later developed bone metastases (Bouizar et al., 1993). We have also demonstrated that transcripts of the 1–139 isoform derived from P2 and P3 were more abundant in tumors that ultimately metastasize, and that P3 transcripts were the most abundant in tumors that metastasized to bone (Bouizar et al., 1999). Expression of tumor-derived PTHrP appears be a crucial factor in a chain of signaling events among the cells of the bone microenvironment that facilitates both the destruction of bone and the growth of tumor cells (Guise et al., 1996; Thomas et al., 1999). In the bone microenvironment, osteoclast-mediated release of TGF-β from the bone matrix is proposed to stimulate high levels of PTHrP gene expression by breast cancer cells (Yin et al., 1999). A recent study of PTHrP promoter activity in the MDA-MB-231 breast cancer cell line has indicated that the P3 promoter is upregulated by TGF-β (Lindemann et al., 2001) The activation of this promoter required an

apparent synergism between smads, components of the TGF-β signaling pathway, and Ets factors (Lindemann et al., 2001). Ets proteins are transcription factors that share the so-called Ets domain, a DNA binding domain that recognizes specifically a GGAA/T-containing DNA sequence. Ets proteins regulate genes that are involved in cell growth and invasion (Sementchenko and Watson, 2000), suggesting that dysregulation of Ets genes may contribute to tumorigenesis (Dittmer and Nordheim, 1998). In particular, Ets1 is overexpressed in a variety of malignant tumors, such as invasive gastric carcinoma, astrocytomas and mammary carcinomas (Nakayama et al., 1996; Valter et al., 1999; Delannoy-Courdent et al., 1996).

In this study we have evaluated PTHrP gene expression in SV40 large T antigen immortalized cell lines derived from normal human breast epithelium (Berthon et al., 1992; Lebeau et al., 1995). Using this model, we have previously demonstrated that PTHrP mRNA and protein expression was increased in the tumorigenic lines as compared to the non-tumorigenic cell line, and PTHrP was demonstrated to stimulate proliferation in an autocrine fashion in the highly tumorigenic cell line (Cataisson et al., 2000). Here we report the splicing pattern of the PTHrP mRNAs for all three cell types. In addition we characterize the mechanisms underlying the differential expression of the peptide, and identify a *cis*-regulatory region and a transcription factor involved in a component of the PTHrP overexpression by the tumorigenic cell line.

2. Materials and methods

2.1. Cell lines and culture

S1T3 and S2T2 cell lines were established from primary cultures of normal human breast epithelial cells from two individuals by immortalization with SV40-T Ag. Like breast epithelial cells in primary culture, the S1T3 cells did not grow in soft agar and did not form tumors in nude mice. In contrast, S2T2, which also did not grow in soft agar, produced slow-growing tumors (<8 mm in diameter 8–10 weeks after inoculation) in nude mice. The NS2T2A1 line was derived from an S2T2 tumor that had been grown in nude mice, re-established in vitro and then repassaged in nude mice. The NS2T2A1 line produced colonies in soft agar and tumors that grew rapidly in nude mice. The epithelial nature of the cell lines was established from 100% staining for pan-cytokeratin and cytokeratin 18. S2T2 and NS2T2A1 cells exhibit specific chromosomal markers resembling those of human breast cancer (Berthon et al., 1992). Cells were cultured in Dulbecco's modified Eagle's and Ham F12 medium (DMEM/F12, 1/1, v/v) with reduced calcium (ATGC, Noisy-le Grand, France) containing 10 mM HEPES, 2 mM glutamine, 10 μg/ml insulin, 5mM cortisol (Sigma, St. Quentin-Fallavier, France), 2 ng/ml epidermal growth factor (Euromedex, Souffelweyersheim, France), 50 IU/ml penicillin, 50 mg/ml streptomycin and 5% Chelex-treated (Sigma) horse serum (ATGC). MDA-MB-231 breast cells lines derived from pleural effusions (Cailleau et al., 1974) were cultured in RPMI medium supplemented with 10% fetal calf serum.

2.2. PTHrP half-life measurement

In experiments to determine the half-life of PTHrP mRNA, confluent cells' medium was replaced by serum-free medium and cells were exposed to actinomycin D (4 μg/ml). RNA's were extracted at timed intervals thereafter.

2.3. Reverse transcription-polymerase chain reaction

RNA extraction was performed as described previously (Bouizar et al., 1993), or total RNA was prepared using the Qiagen RNeasy kit by following the manufacturer's instructions. After treatment with DNase I, RNA (5 μg) was transcribed into cDNA using MMLV(H-)-RT (Promega). The cDNA was then used for polymerase chain reaction (PCR).

2.3.1. Semiquantitative polymerase chain reaction for PTHrP transcripts

The strategy for primer design and use was based on the sequences of the PTHrP gene (Yasuda et al., 1989; Southby et al., 1995, Fig. 1A and B). RT-PCR was performed as described previously (Bouizar et al., 1993) using 1 μg of total RNA. Complementary DNA was amplified using 0.5 U *Thermophilus aquaticus* polymerase (Appligene, Illkirch, France) in buffer containing 25 pmol of each specific sense and antisense primer. Amplification was performed in a Techne PHC-3 thermal cycler with denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s. The number of RT-PCR cycles was optimized for semiquantitative assessment of the product by generating saturation curves of the amounts of RT-PCR products against the number of cycles for 0–45 cycles of amplification (data not shown). The amplification profile adopted was 23 cycles for S14, 25 cycles for 3′ alternative splicing cDNA and 30 cycles for the 5′ exons' cDNA amplification. Oligonucleotides utilized for PCR and authentication of PCR products were selected using GeneWorks (Intelligenetics Inc., Mountain View, CA) and purchased from Genosys (Cambridge, UK). The sequences of the primers are reported in Fig. 1B. PCR reactions were carried out using the internal control gene S14 (human small ribosomal protein 14) which is ubiquitously expressed at constant level and allows the

relative quantitation of PTHrP mRNA (Bouizar, et al., 1993). The resulting PCR products were resolved by electrophoresis in a 2% agarose gel. DNA was transferred to a Genescreen nylon membrane (NEN, Paris, France) by capillary action, hybridized overnight at 42 °C in hybridization solution containing 500 000 cpm of a (α^{32}P) dATP-labeled oligonucleotide probe. The sequences for oligonucleotide probes were:

IA AS	5′-AGGCAATTATTAGAAAGCAGGTACCTCTTCCAAGCTGC-3′
IC AS	5′-GAGGTTGAAAACCGAGCGGAGGAATGTTCACACGCTCCGAGGCAAAC-3′
II AS	5′-GCATCAATGATAAGTAGTGTGTTTACACGTCTCCCATAGCAATGTC-3′
IV AS	5′-GTTTCCTGAGTTAGGTATCTGCCCTCATCATCAGACCCAAATCGGACGG-3′
S14 AS	5′-CCCGGAGTTTGATGTGTAGGGCGGTGATACCCAGCTCCTTGCACCTCTGG-3′

The autoradiography signals were analyzed with a densitometric scanner (Transidyne General Corporation, Roucaire, Vélizy-Villacoublay, France), and the results were expressed in arbitrary units as the ratio of PTHrP-amplified cDNA to S14 densitometric values.

2.3.2. Analysis of Ets transcription factor expression

The RT-PCR reaction mix (25 μl) contained 1 unit of RedTaq™ Polymerase (Sigma) and 500 nM of each primer of a primer pair as listed below (Dittmer et al., 1997). The DNA was amplified by 35 PCR cycles which included denaturing at 95 °C for 30 s, annealing at 55 °C for 1 min, and polymerization at 72 °C. Amplification products were detected by staining with ethidium bromide after separation on a 2% agarose gel.

The following primers were used:

Ets-1(410 bp)	Forward: 5′-ATTCAGCCTGAAAGGTGTAGA-3′
	Reverse: 5′-AGTAGTCATTCTGCAAGGTGTC-3′
Ets-2 (730bp)	Forward: 5′-GCT ACC TTC AGT GGC TTC AA-3′
	Reverse: 5′-TTG GCT TAT TGA GGC AGA GA-3′
Ese-1 (328 bp)	Forward: 5′-ACTGATGGCAAGCTCTTCCC-3′
	Reverse: 5′-GGCTCAGCTTCTCGTAGGTC-3′
GAPDH (414bp)	Forward: 5′-CACTGACACGTTGGCAGTGG-3′
	Reverse: 5′-CATGGAGAAGGCTGGGGCTC-3′

2.4. SDS–PAGE western blot analysis

Nuclear extracts were prepared as previously described (Blumenthal et al., 1999). Briefly, after washing the cell layer with PBS, the cells were scraped off and transferred to an Eppendorf tube. Cells were resuspended in 400 μl hypotonic buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF), incubated at 4 °C for 15 min and lysed by addition of Nonidet P-40 and vortexing for 10 s. After centrifugation at 13 000 rpm for 30 s, pellets were resuspended in 100 μl buffer C (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1mM DTT, 0.5 mM PMSF). After rocking for 15 min, the protein solution was cleared by centrifugation and stored at −80 °C. Total protein amount was measured by using the Bio-Rad Bradford reagent. For Western blot analysis, 30 μg of nuclear protein were subjected to electrophoresis in a 10% SDS polyacrylamide gel, blotted onto a PVDF-membrane (Millipore) and incubated with anti-Ets1 or Ets2 antibody (Santa Cruz Biotechnology, Inc.) essentially as described previously (Lindemann et al., 2001).

2.5. Construction of the PTHrP-Luc plasmids

The PTHrP-luciferase reporter gene constructs were derived from a group of human PTHrP-CAT constructs (Vasavada et al., 1993), using the designated restriction sites and subsequent ligation into pGL-2 (Promega, USA). The 4.3 kb construct containing all three promoters and the 5′BamH I-Hind III isolated P1 construct were cloned from a 4.3 kb 5′BamH I-Hind III CAT construct. The Acc I-Hind III construct containing P3 in isolation was cloned directly from the Acc I-Hind III CAT construct. The Avr II-Acc I construct containing P2 in isolation as well as the Bgl I-Hind III construct containing P3 in isolation were cloned from a BamH I-Hind III CAT construct. When possible, PTHrP fragments were cloned into compatible sites within the pGL-2 polylinker; otherwise, sites were blunted with Klenow or T4 polymerase. The Ets1

binding site (EBS-1) site mutation in the Bgl I-Hind III construct containing P3 in isolation was mutated with the 'QuickChange' kit (Stratagene, LaJolla, CA) and the following primers: (Sense: GGAATCAACTTTCCG-GTTGCAACCAGCCC; Antisense: GGGCGTGTTG-CAACCGGAAAGTTGATTCC). These primers abolished a BspE1 restriction enzyme site. In all cases, mutagenesis was confirmed by DNA sequencing performed by the University of Chicago Cancer Research Center DNA Sequencing Facility. The P2/P3 luciferase reporter construct as well as mutated EBS-1 and Sp1 site derivatives along with pCDNA-3-(Invitrogen, Carlsbad, CA) based expression vectors that drive chicken Ets1 and Ets2 cDNAs are described in Lindemann et al. (2001).

2.6. Transient DNA transfections and analysis

For the transfection of Luciferase constructs, cells were plated at a density of 200 000 cells/well in twelve-well plates, 2 μg of Luc reporter and 0.2 μg of pSV β-galactosidase control vector were identically co-transfected using TransFast (Promega, France). Cotransfections were performed with 1 μg reporter plasmid, 1 μg effector plasmid and 0.2 μg of pSV β-galactosidase. Baseline expression of the reporter construct was established by cotransfecting the pCDNA-3 vector that did not have an effector cDNA added. The medium was changed the following day, and cells were harvested 48 h post-transfection in reporter lysis buffer (Promega, France), after which cellular debris was pelleted. For luciferase activity determination, 20 μl of cell extract were used in a reaction mixture that contained beetle luciferin (Promega), and luciferase activity was measured in a luminometer. The chemiluminescent substrate system for β-galactosidase measurement was purchased from Boehringer Mannheim, and the results were used to normalize for transfection efficiency. Data represent mean ± S.D. or SEM of three separate wells of cells transfected in parallel. Each transfection was repeated at least two times in cells derived from independent passages.

2.7. Statistical methods

Data were analyzed by SPSS software and significance values assigned through Student's t-test. $P < 0.05$ was considered to be significant. All data represent mean of at least three values and error bars indicate SEM or S.D. depending on whether the values were derived from separate experiments or a single experiment.

3. Results

3.1. NS2T2A1 expresses increased levels of mRNA for PTHrP 1–139

In the following studies, we have employed three cell lines (S1T3, S2T2 and NS2T2A1) derived from SV40 large T antigen immortalized breast epithelial cells. In tumorigenicity assays using nude mice, S1T3 cells did not produce tumors, S2T2 produced small slow-growing tumors, while the third line, NS2T2A1 (originally derived from a xenografted S2T2 tumor), produced aggressive tumors. The characteristics of these cells and the chromosomal abnormalities they contain suggested that they could provide an in vitro model of breast tumor progression (Berthon et al., 1992). This model provides diploid breast epithelial cells of differential tumorigenicity and growth characteristics, derived from the same donor, permitting comparisons of gene expression that may reflect the acquisition of invasive characteristics. Previously we had reported that greater PTHrP gene expression correlated with increased tumorigenicity among the S1T3, S2T2 and NS2T2A1 lines; therefore, we undertook a series of studies to determine the mechanisms which underlie differential expression of this calcitropic factor. As a first step to evaluate PTHrP gene expression, total RNA was extracted from confluent cultures of each of the cell lines and subjected to the sensitive semiquantitative RT-PCR technique to determine total PTHrP mRNA expression as well as the specific 3′ splicing variant expressed. Relative levels of total PTHrP mRNA were determined by using primers to exon IV, common to all transcripts, and primers specific for the S14 mRNA, a ubiquitously expressed control gene that permits the semiquantitative determination. As shown in Fig. 2A and B, increased levels of PTHrP mRNA corresponded with the increased tumorigenic potential of the cell lines. To address which of the PTHrP protein isoforms were produced and if they were differentially expressed, the 3′ alternative splicing patterns were investigated by amplifying transcripts containing exons V, VI, and VII (corresponding to the 1–139, 1–173 and 1–141 mRNAs respectively). Transcripts containing exon V (encoding PTHrP 1–139) (Fig. 2A and C) were present in all three cell lines, but were substantially increased in the highly tumorigenic cell line NS2T2A1. Transcripts containing exon VI (encoding PTHrP 1–173) (Fig. 2A and D) were detected only in the NS2T2A1 line. Exon VII transcripts (corresponding to PTHrP 1–141) (Fig. 2A and F) were expressed at similar levels in all three cell lines. Overall, it appeared that increased PTHrP expression in the highly tumorigenic cell line NS2T2A1 is associated with increased expression of transcripts encoding for PTHrP 1–139.

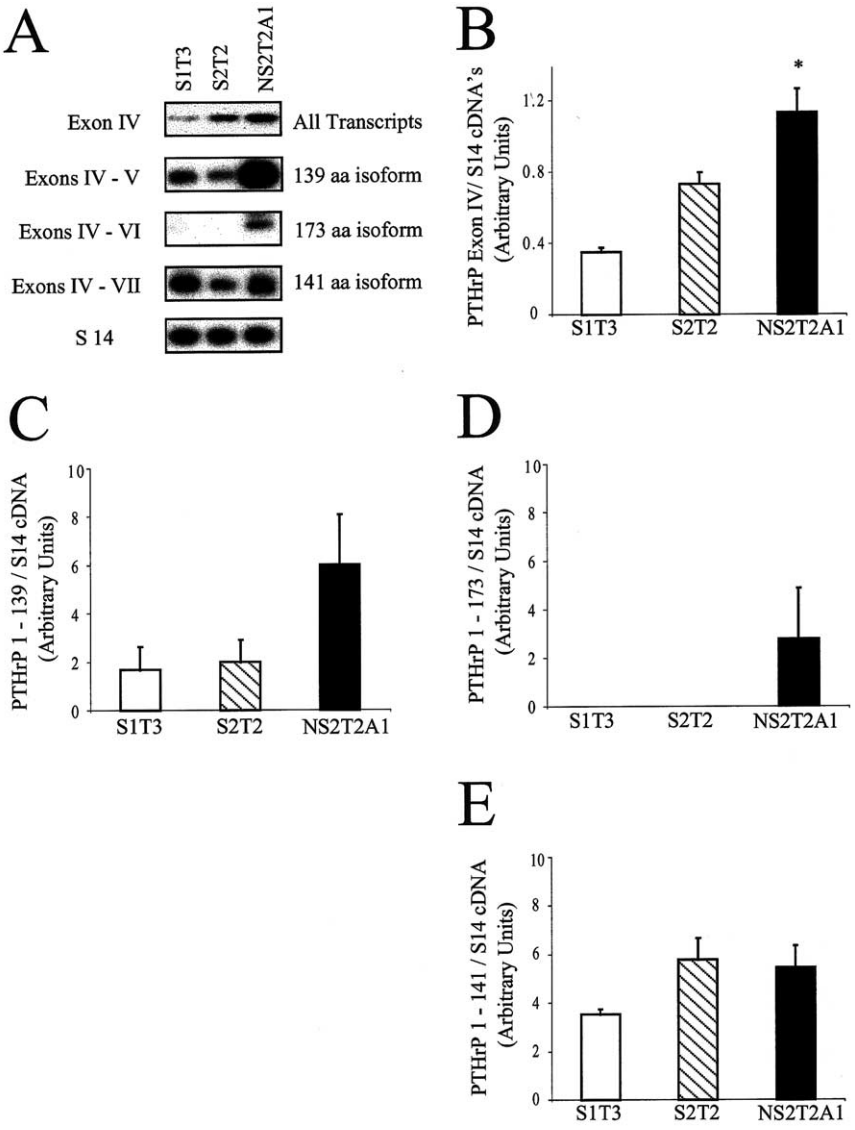

Fig. 2. Semiquantitative RT-PCR analysis of 3′ PTHrP mRNA splicing patterns in S1T3, S2T2 and NS2T2A1 cells. *Panel A:* PCR amplification of PTHrP cDNA was performed between exons IV and V for PTHrP 1–139, exons IV and VI for PTHrP 1–173 and exons IV and VII for PTHrP 1–141. PCR products were resolved on a 2% agarose gel, transferred to a nylon membrane and hybridized with an internal oligonucleotide. *Panels B–E:* expression of the various PTHrP transcripts relative to S14 transcripts, evaluated by densitometry. Each bar represents the mean and standard error of the mean of densitometry readings made from PCR reactions performed on cDNA derived from three separate experiments. (B) all transcripts. (C) transcripts that encode the 1–139 isoform. (D) transcripts that encode the 1–173 isoform. (E) transcripts that encode the 1–141 isoform. * $P < 0.05$ vs S1T3.

3.2. PTHrP mRNA stability analysis

In order to determine if the altered expression of PTHrP mRNAs was due to changes in PTHrP mRNA stability, we measured the half-life of the PTHrP transcripts. RNA synthesis was blocked by actinomycin D, and PTHrP mRNA expression was determined by semiquantitative RT-PCR with primers specific to the common exon IV. Under these conditions, where all transcripts are evaluated together, no significant differences in PTHrP mRNA stability could be detected among the three cell lines (Fig. 3). Thus it appeared that the half-life of PTHrP mRNA was not increased in the tumorigenic cell lines that contain a greater abundance of PTHrP transcripts.

3.3. PTHrP promoter usage

The half-life studies suggested that the increased PTHrP mRNA expression observed in the S2T2 and NS2T2A1 lines was the result of increased PTHrP transcription. To determine if the increased PTHrP

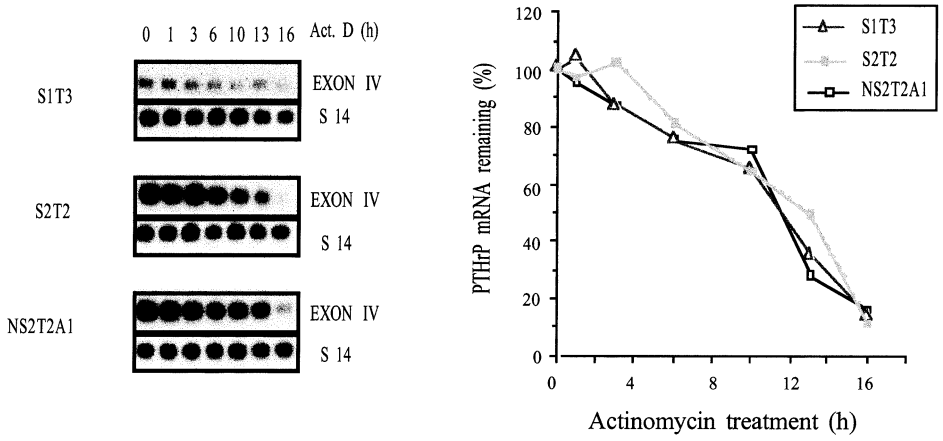

Fig. 3. Stability of total PTHrP mRNA species in S1T3, S2T2 and NS2T2A1 cells. Cells were treated with vehicle alone or actinomycin for time indicated. On left panel, exon IV common to all transcripts was amplified using the optimal number of cycles for semiquantitative analysis. PCR products were confirmed by hybridization with ^{32}P-labeled probe for exon IV or S14. On right panel, the intensities of autoradiograph signals were quantitated and the percentage of PTHrP mRNA remaining at each time point compared to the zero time point was plotted after normalization to the signal for S14 for the same lane. This figure represents data from a single experiment that was repeated three times with similar results.

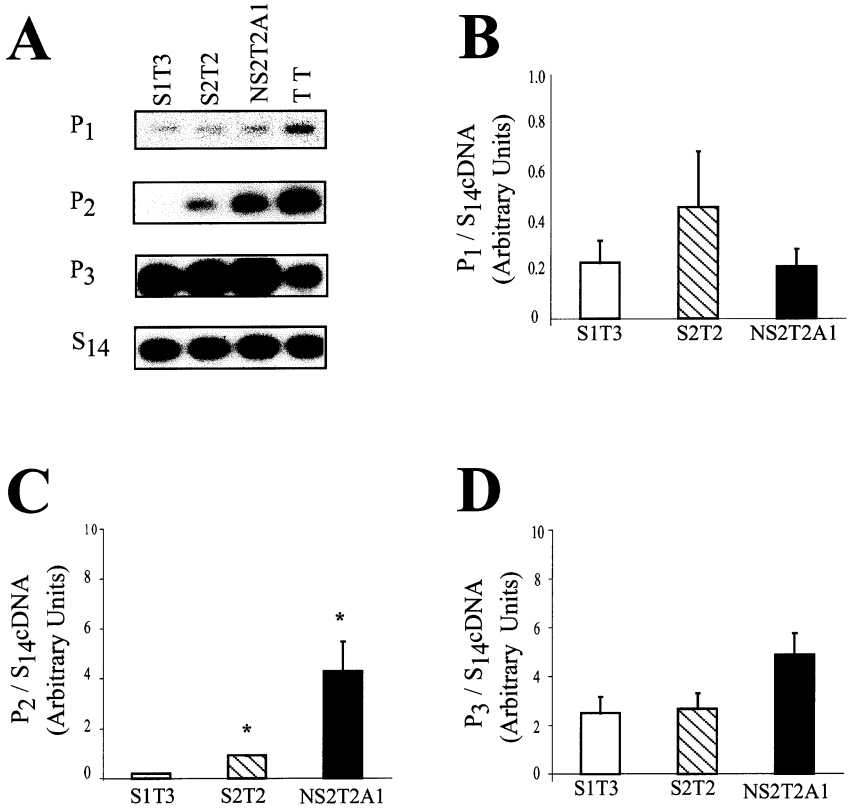

Fig. 4. Semiquantitative RT-PCR analysis of PTHrP promoter usage in S1T3, S2T2 and NS2T2A1 cells. *Panel A:* cDNA's from each cell line were amplified with promoter-specific primer sets. PCR products were resolved on a 2% agarose gel, transferred to a nylon membrane and hybridized with an internal oligonucleotide. TT represents a medullary thyroid carcinoma control cell line that expresses high levels of PTHrP derived from all three promoters. *Panels B-D:* expression of the various PTHrP transcripts relative to S14 transcripts, evaluated by densitometry. Each bar represents the mean and standard error of the mean of densitometry readings made from PCR reactions performed on cDNA obtained from three separate experiments. If an error bar is not present it indicates the error was not of sufficient magnitude to be illustrated on the graph. (C) P2-derived transcripts. (D) P3-derived transcripts * $P < 0.05$ vs S1T3.

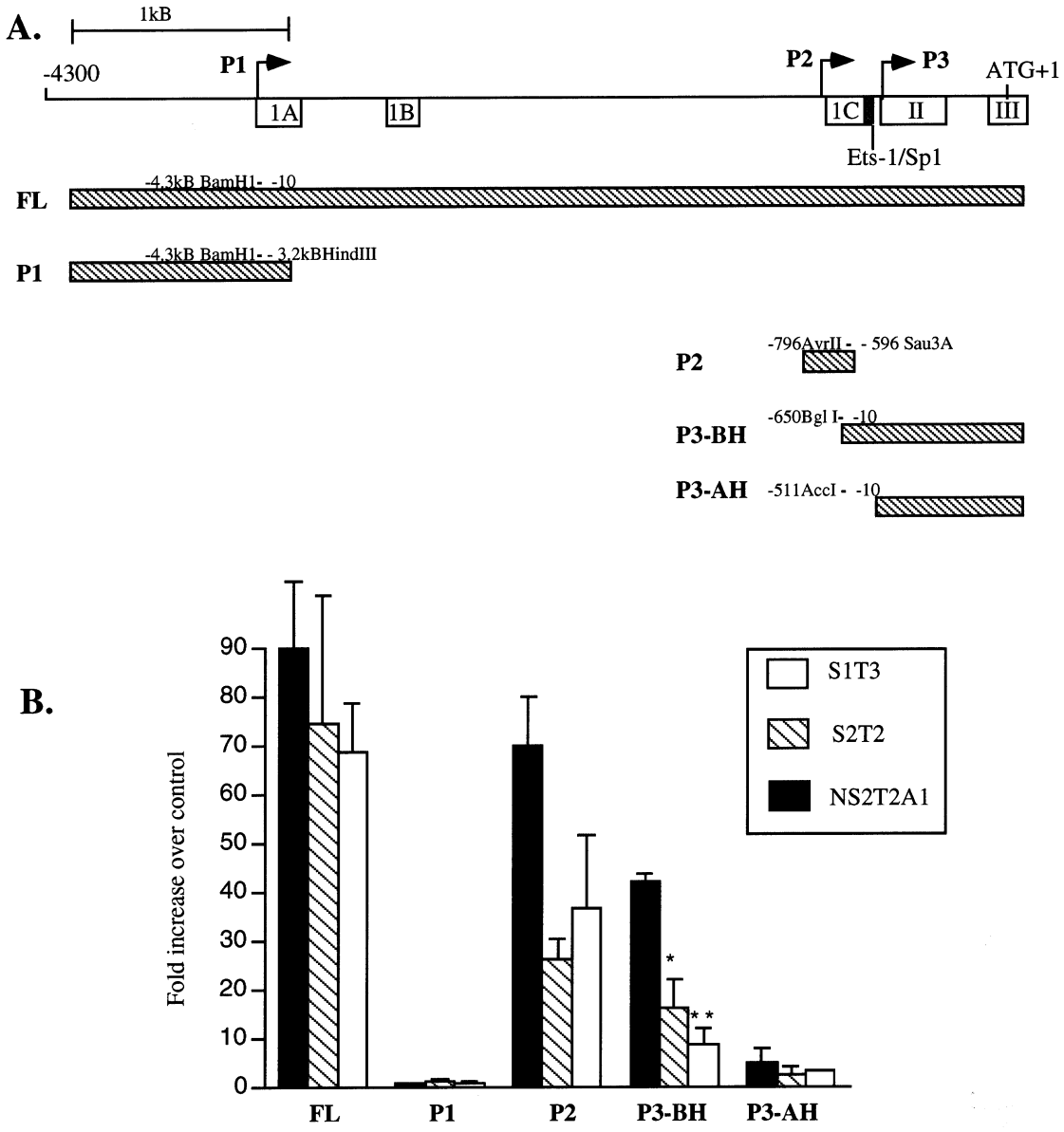

Fig. 5. Expression of reporter genes driven by 5′PTHrP sequences in S1T3, S2T2 and NS2T2A1 cells. *Panel A:* a schematic representation of the 5′-flanking region of the human PTHrP gene and the 5′PTHrP-Luc constructs used in the analysis: exons are indicated by boxes, promoters by arrows and the shaded region represents the region of the EBS-1 and Sp1 sites. The construct represented as FL contains 4.3 kb of upstream sequence and all three promoters. P1 contains the P1 promoter in isolation. P2 contains the P2 promoter and minimal sequence upstream or downstream. P3-BH contains 140 bp upstream of the P3 TATA including the Ets-1/Sp1 site and the downstream non-translated exon II. P3-AH contains only 15 bp of upstream sequence, thus eliminating the Ets-1/Sp1 site, but contains downstream sequences. *Panel B:* the bar graph depicts reporter gene activity normalized to the activity of a promoterless luciferase vector pGL-2. Bars represent the average of three samples and error bars indicate SEM. If an error bar is not present it indicates the error was not of sufficient magnitude to be illustrated on the graph. Every construct was transfected into each of the three lines in three separate experiments and similar results were obtained. * represents $P < 0.05$ NS2T2A1 vs S2T2; ** represents $P < 0.01$ NS2T2A1 vs S1T3.

mRNA expression was due to increases in transcription from a specific PTHrP promoter, RT-PCR was initially carried out using primers to exons adjacent to the promoter P1 (exon IA), P2 (upstream of the 5′ end of exon IC) and P3 (exon II) as well as reverse primers to common exon III. Low levels of a single 610 bp promoter 1-derived transcripts were detected in all cell lines (Fig. 4A and B). P2-initiated transcripts were detected only in the S2T2 and NS2T2A1 lines (Fig. 4A and C). P3-initiated transcripts were abundantly expressed in RNA from each cell line, in particular in NS2T2A1 (Fig. 4A and D).

3.4. Increased PTHrP P3-driven reporter gene activity in NS2T2A1 cells

In an effort to identify *cis*-acting DNA elements that control PTHrP gene expression in these cell lines that might influence the transcriptional regulation of this factor, cell lines were transiently transfected with a series of reporter constructs driven by 5′PTHrP sequences. The results of these transient transfections are graphically represented relative to the activity of the empty pGL-2 vector in all the lines (Fig. 5B). The isolated promoter 1 construct (P1) produced very little reporter gene activity in all of the cell lines, and it is unclear whether this result reflected the low levels of endogenous activity of this promoter or that this construct lacks appropriate activating sequences. As shown in Fig. 5B, both the full length (FL) construct and the promoter 2 (P2) construct containing a minimal (30 bases) sequence upstream of the transcription initiation site produced relatively high levels of reporter gene activity in the three lines. Even the S1T3 line, which contained very low levels of endogenous P2-derived transcripts, produced substantial P2-driven luciferase activity. Subsequent experiments indicated that differential activity of P2 in these immortalized breast epithelial lines was dependent on the methylation status of sequences in intron 2 (Bouizar, unpublished observations). This finding limits the utility of larger PTHrP P2 containing reporter constructs with these specific lines. In contrast, reporter gene activity from an isolated promoter 3 construct that contained 140 bp of upstream sequence (P3-BH) reflected endogenous P3 activity in that a progressive increase of luciferase activity was observed in the S2T2 and NS2T2A1 cells. As shown in Fig. 5B, deletion of 125 bp of sequence (including the Ets-1/Sp1 sites) upstream of P3 reduced reporter gene activity in each of the lines (90% in NS2T2A1 and S2T2, 63% in S1T3) to approximately baseline levels. This suggested that the differential P3 reporter gene activity observed in the immortalized cell lines was controlled by *cis*-acting sequences within a 125 bp region.

3.5. The Ets1 binding site is required for high levels of P3 promoter activity

Previous work with T-cell lines had suggested that activity of the PTHrP-P3 promoter was largely dependent on a sequence 24–44 bp upstream of the TATA box located at the end of exon 1C which contained Ets1 and Sp1 sites (Holt et al., 1993; Dittmer et al., 1997). To evaluate the role of this EBS 1 and Sp1-binding motifs in the transactivation of the P3 promoter in the cell lines, we employed a series of PTHrP reporter constructs where these sites had been altered by site-directed mutagenesis. As shown in Fig. 6B and C, Mutation of the EBS-1 site reduced activity of the P3 containing reporter constructs 50–70% in the NS2T2A1 and S2T2 lines. Mutation of the Sp1 site also reduced reporter gene activity of the P2/P3 construct by ~40% in these lines. In the S1T3 line, mutation of EBS-1 and Sp1 sites reduced activity of the constructs ~40 and 30% respectively (6D). Taken together these findings suggest that the EBS-1 and Sp1 sequences are crucial to provide full activity of P3 containing reporter genes in all the breast epithelial cell lines.

3.6. Increased levels of Ets1 protein is expressed in NS2T2A1 cells as compared to S2T2 and S1T3 cells

Next we examined the expression pattern of Ets proteins in the three lines. We focused on Ets1, Ets2 and Ese-1/Esx. These Ets proteins have been reported to be capable of activating the PTHrP P3 promoter (Dittmer, et al., 1993; Karperien et al., 1997; Lindemann et al., 2001) and to be expressed in a variety of breast cancer cell lines (Blumenthal et al., 1999; Watabe et al., 1998; Chang et al., 1997; Oettgen et al., 1997). By using RT-PCR, we observed that the level of Ese-1/Esx RNA was similar in all the three cell lines (Fig. 7A). In contrast, the levels of Ets1 and Ets2 mRNA were found to be increased in the invasive NS2T2A1 cells as compared with the levels S2T2 and S1T3 cells (Fig. 7A). Despite this slight difference in Ets1 RNA expression, the difference in the Ets1 protein levels among the lines was dramatic. As judged by Western blot analysis, little Ets1 protein could be detected in nuclear extracts derived from S2T2 and S1T3 cells, while an Ets1-antibody immunoreactive band was clearly visible in nuclear extracts from NS2T2A1 cells (Fig. 7B). As a positive control for an Ets1-expression in a breast cancer cell line, the highly invasive MDA-MB-231, was used. Specific antibody analysis showed that little Ets2 protein could be detected in nuclear extracts derived from S1T3, S2T2, NS2T2A1 or MDA-MB-231 cells (Fig. 7B).

3.7. Exogenous Ets1 expression can activate PTHrP reporter genes in NS2TSA1 cells

Increased PTHrP P3-mediated activity in NS2T2A1 cells is accompanied by the acquisition of Ets1 and Ets2 mRNA and increased Ets-1 protein expression. We reasoned that Ets1 or Ets2 could potentially trigger increased levels of PTHrP gene expression in the NS2T2A1 cells. To test this hypothesis, all three cell lines were cotransfected with PTHrP reporter genes as well as expression vectors that produced Ets1 or Ets2 proteins. As shown in Fig. 8A, expression of Ets1 but not Ets2 was capable of activating constructs containing either P3 in isolation (P3) or as part of 4.3 kb of FL construct in NS2T2A1 cells. Mutation of the EBS-1 binding site prevented activation by exogenous Ets1 in this assay (P3EM in Fig. 8A). Expression of Ets1 or Ets2

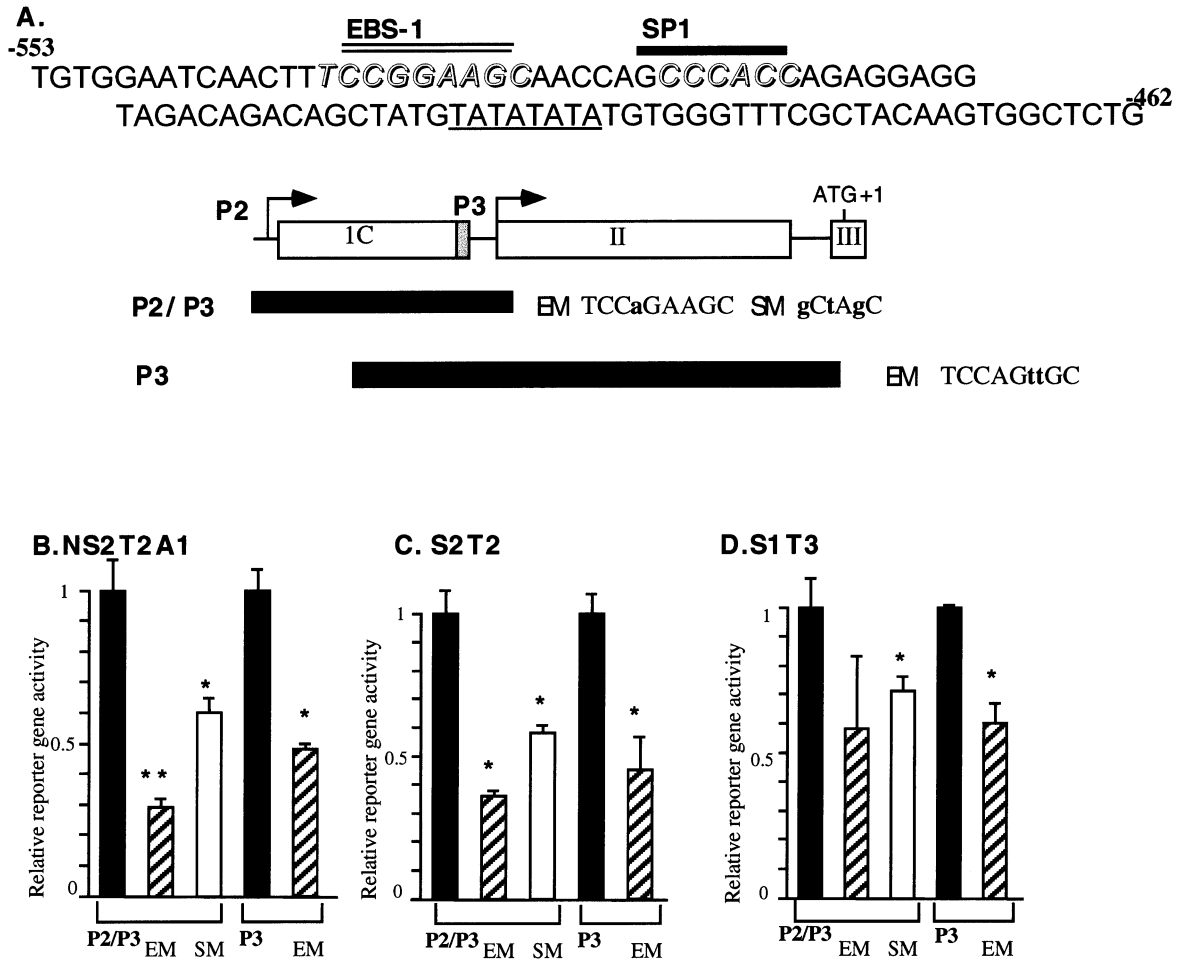

Fig. 6. Mutation of Ets1 and Sp1 binding sites reduces PTHrP reporter gene activity. *Panel A:* sequence ranging from −553 (top line left) to −462 (second line right) of 3′ end of exon 1C and intron region to the transcription start site for the P3 promoter. The sequences of the EBS-1 (double overlined) and Sp1 (bold overlined) sites are indicated in shadowed italics and the P3 TATA box is underlined. Below the sequence is a schematic of the portion of the 5′PTHrP gene sequences contained in the reporter constructs that were subjected to site directed mutagenesis. The arrows indicate transcription initiation sites, exons are indicated by boxes and the shaded region corresponds to the region of the EBS-1 and Sp1 Sites. The P2/P3 construct encompasses sequences from −792 Sty-1 site to −446. The P3 construct encompasses sequences from the −650 Bgl-1 site to −10. To the left of the promoter schematics are the mutated sequences for the EBS-1 (EM) and the Sp1 (SM) site with altered nucleotides indicated in lower case bold letters. Panels B-D: the panels represent relative reporter gene activities of the promoter constructs and mutated derivatives in the three transformed breast epithelial cell lines. The parental constructs, EBS-1 mutated and Sp1 mutated constructs are indicated by solid, stippled and open bars respectively. Reporter gene activity for each of the constructs and their mutated derivatives is expressed as a fraction of that of the individual parental constructs (P2/P3, or P3) that has been arbitrarily set to 1. The panels represent average of three replicates and error bars indicate SD from a single experiment. Each experiment was repeated three times and produced similar results. * represents $P < 0.05$ vs the parental construct; ** represents $P < 0.01$ vs the parental construct.

failed to activate PTHrP reporter gene expression in the less tumorigenic S2T2 and non-tumorigenic S2T3 cells (Fig. 8B and C), indicating that there is an additional factor or factors associated with the NS2T2A1 line that facilitates activation of PTHrP reporter gene expression by the Ets1 transcription factor. Thus the acquisition of Ets-1 expression and its apparent ability to activate PTHrP gene expression correlates with the increased tumorigenicity of NS2T2A1 cells.

4. Discussion

In this study we report that the pattern of PTHrP promoter usage and 3′ alternative splicing from the SV-40 T antigen immortalized breast epithelial cells parallels that exhibited by the primary breast cancers (Bouizar et al., 1993). In primary breast cancers high levels of P3-initiated transcripts were positively correlated with the development of metastasis as well as

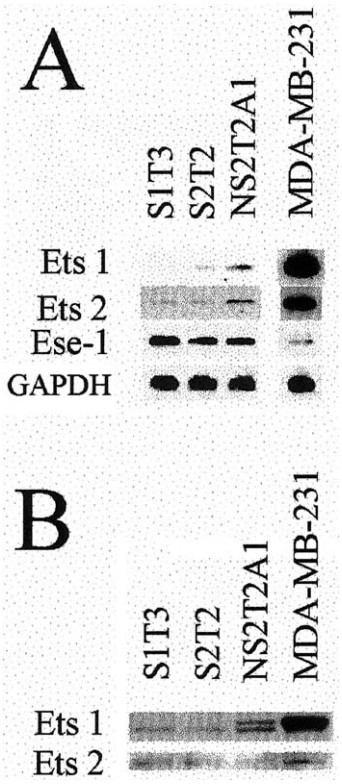

Fig. 7. Ets factor expression in S1T3, S2T2 and NS2T2A1 cells. *Panel A:* Ets factor mRNA expression relative to GADPH was evaluated by RT-PCR. Expression was compared to the established breast cancer line MDA-MB-231 where Ets factor expression has been extensively studied (Lindemann et al., 2001). All of the lines expressed Ese-1 transcripts, but NS2T2A1 cells appeared to contain increased levels of Ets2 and Ets1 mRNA. *Panel B:* Western blots performed with specific antibodies to Ets-1 and Ets-2. Ets-2 protein expression levels were minimal among the three breast epithelial cell lines; however, there was increased intensity of Ets-1 immunoreactivity in the NS2T2A1 line. There appeared to be an immunoreactive band of slightly decreased mobility in the NS2T2A1 extract that co-migrated with the majority of this protein that is present in the MDA-MB-231 line.

metastasis to bone (Bouizar et al., 1999). Finally, increased levels of transcripts that encode the 1–139 amino acid isoform were positively correlated with metastasis to bone (Bouizar et al., 1999). The tumorigenic NS2T2A1 cells contained increased levels of the transcripts that encode for the 1–139 isoform. In general, the pattern of alternatively spliced PTHrP mRNA's expression in the immortalized breast epithelial cells was similar to that in established breast cancer cell lines, T-47D, MCF-7 and MDA-MB-231 (Southby et al., 1995). The pattern of PTHrP transcripts observed in tumors as well as in the immortalized breast epithelial cells reflects steady-state mRNA levels. This could result either from increased transcription from specific PTHrP promoters or increased mRNA stability resulting from a specific 5′ or 3′ sequence. The RNA half-life analysis suggested that the overall differences in PTHrP coding region mRNA levels observed in the immortalized breast epithelial cell lines did not result from variance in message stability; however, this finding did not exclude the possibility that there were alterations in the stability of specific PTHrP isoforms among the three cell lines.

High levels of PTHrP gene expression in certain neoplasms appear to result from the activity of specific PTHrP promoters. In adult T cell leukemia cells infected with the HTLV-1 virus, high levels of PTHrP expression have been attributed to the activation of the P3 promoter (Holt et al., 1993; Southby et al., 1995). In squamous carcinoma cell lines that express very high levels of PTHrP and produce hypercalcemia when grown in nude mice, the majority of PTHrP transcripts appeared to be derived from the P1 and P3 promoters (Wysolmerski et al., 1996; Foley et al., 1996). A study of several cancer cell lines has suggested that the P3 promoter was most active in lines that produced high levels of PTHrP mRNA (Southby et al., 1995).

Our transient transfection experiments provided some insight into how the P3 promoter is differentially regulated among the three cell lines. Transfection of P3 in isolation along with 140 bp of upstream sequences produced reporter gene activity that roughly approximated the endogenous activity of this promoter in each of the cell lines. Deletion of 125 bp of sequences upstream of P3 (including the EBS-1 and Sp1 sites) abolished reporter gene activity in all three lines. These findings are consistent with several other reports emphasizing the importance of the Ets/Sp1 region in controlling PTHrP gene expression in physiological and pathological circumstances (Dittmer et al., 1993; Karperien et al., 1997; Foley et al., 1999). The activity of reporter gene constructs that contained mutations in the EBS-1 and Sp1 site was substantially reduced in the tumorigenic S2T2 and NS2T2A1 cells. This sequence has been reported to mediate Tax gene-activated gene expression of human PTHrP constructs in T cell lines and retinoic acid-induced stimulation of the murine PTHrP gene in embryonal carcinoma cells (Dittmer et al., 1993; Karperien et al., 1997) Also, this sequence was reported to be crucial to the production of high levels of reporter gene activity in a murine keratinocyte cell line (Foley et al., 1999). Finally these sites appear to act synergistically with smads as part of TGF-β-induced activation of PTHrP reporter constructs in the breast cancer cell line MDA-MB 231 (Lindemann et al., 2001).

The acquisition of Ets1 expression by many tumors is correlated with an invasive phenotype, suggesting that the trans-acting factor may regulate genes that facilitate metastasis (Dittmer and Nordheim, 1998; Nakayama et al., 1996). In general, putative breast cancer cell lines characterized as being highly invasive on the basis of in vitro assays, such as MDA-MB-231, express high levels of Ets1 and PEA-3 and relatively low levels of Ets2 and

Ese-1/Esx (Gilles et al., 1997; de Launoit et al., 2000). Lines with low invasive potential (MCF-7 Zr-75-B) appear to express substantial mRNA or protein levels of Ets2 and Ese-1/Esx (Gilles et al., 1997; de Launoit et al., 2000). All three of the immortalized breast epithelial lines in this study expressed Ese-1/Esx, but the highly tumorigenic NS2T2A1 line contained increased levels of Ets1 mRNA, suggesting that these factors may be responsible for the increased EBS1-dependent PTHrP reporter gene activity in these cells. The cotransfection experiments indicated that increased EBS1-dependent PTHrP reporter gene activity could be induced by the Ets1 but not the Ets2 expression vector, suggesting that endogenous Ets1 could be a factor leading to increased PTHrP production observed in NS2T2A1 cells. As was previously reported for low-PTHrP-producing MCF-7 cells (Lindemann et al., 2001), ectopic expression of Ets-1 was not sufficient to activate PTHrP reporter gene expression in the S2T2 and S2T3 lines. Thus it appears that NS2T2A1 cells possess an additional factor or factors required for Ets1-mediated activation of PTHrP reporter gene expression.

At this time, a role for Ets1 in the pathogenesis of human breast cancer has not been clearly defined. Surveys of invasive tumors derived from breast tissue detected some Ets1 mRNA and protein in carcinoma cells, but transcripts and immunoreactivity were also observed in the fibroblasts and endothelial cells of the stroma (Behrens et al., 2001). The Ets factors Esx and PEA-3 also have been observed in carcinoma cells of invasive breast cancers, suggesting that these factors may influence the pathogenesis of the disease (Watabe et al., 1998; Chang et al., 1997; de Launoit et al., 2000). Further studies focusing on Ets factor expression and its relationship to PTHrP gene expression may provide mechanistic insights into the progression of breast cancer.

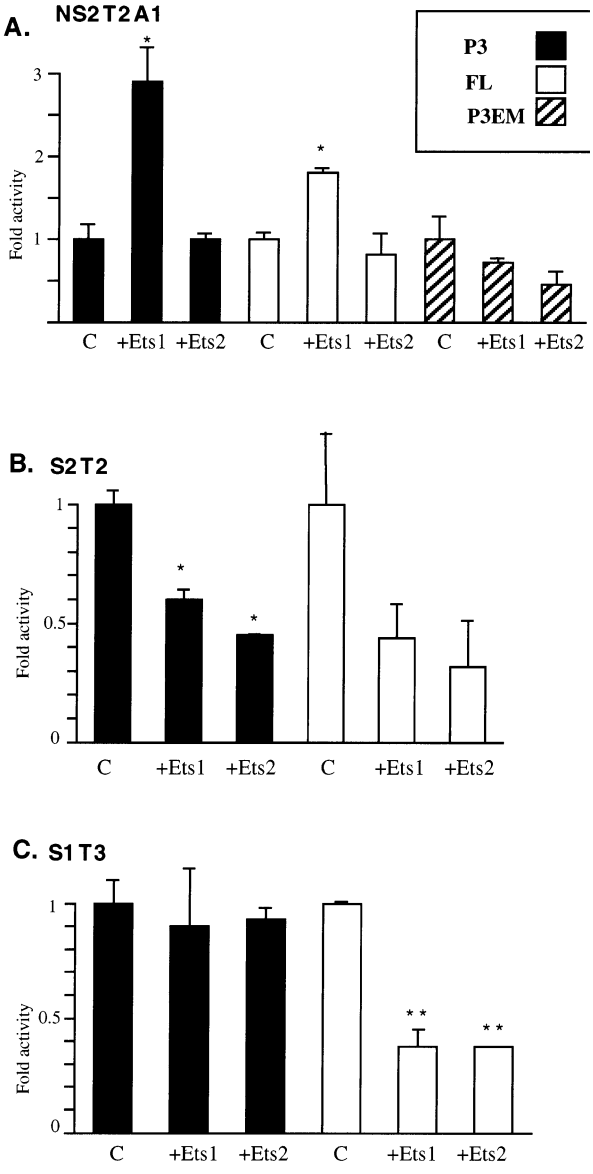

Fig. 8

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Fig. 8. Overexpression of Ets-1 activates PTHrP reporter gene expression in the NS2T2A1 but not S2T2 and S1T3 cells. Each of the three lines was cotransfected with a series of PTHrP reporter genes and expression vectors for Ets1, Ets2 or the parental vector pCDNA-3 (used to establish baseline activity of the construct). The bars in each panel represent fold activity relative to the parental reporter construct that was arbitrarily set to one. Solid, empty or stippled bars represent reporter gene activity from the P3 (−650 Bgl-I-10) FL (full-length −4300 BamH I-10) and P3EM (−650 Bgl-I-10 with EBS-1 site mutated) respectively. The panels represent average of three replicates; error bars indicate S.D. from a single experiment. Each experiment was repeated three times and produced similar results. If an error bar is not present it indicates the error was not of sufficient magnitude to be illustrated on the graph. In panel A, cotransfection of NS2T2A1 cells with an Ets1 expression vector increased reporter gene activity of the P3 promoter and the full-length construct ~2.8-and 1.8-fold respectively. Cotransfection of the Ets1 expression vector with a P3 reporter gene with a mutated EBS-1 site failed to increase reporter gene activity. The Ets2 expression vector failed to increase PTHrP reporter gene activity. In panels B&C both Ets1 and Ets2 expression vectors failed to increase reporter gene activity derived from any of the constructs, including the P3EM (not shown). Both the Ets1 and Ets2 expression vectors appeared to repress reporter gene activity in the S2T2 and S1T3 cells. * represents $P < 0.05$ vs the parental construct; ** represents $P < 0.01$ vs the parental construct.

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