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13. ABSTRACT *(Maximum 200 Words)*

The growth factor pleiotrophin (PTN) is a positive regulator of tumor angiogenesis. PTN is expressed in breast cancer cell lines and primary breast tumor specimens and has been discussed as one major factor for the malignant phenotype of human mammary carcinoma. The expression of PTN is regulated by at least two promoters, one that is species-conserved and one that is human-specific. The latter is generated by insertion of a human endogenous retrovirus-like element (HERV) into the PTN gene. Activity of the HERV promoter element results in the transcription of HERV-PTN fusion transcripts in addition to PTN transcript. The HERV-PTN fusion transcripts are expressed in human breast cancer cell lines, in primary human breast tumor specimens, in epithelial cells of benign breast specimens and in some epithelial and myoepithelial cells of normal breast tissue. The expression of PTN in normal and pathologic human breast tissue is mediated by coexpression of the species-specific transcript and the HERV-PTN fusion transcript. Here we report the identification of various cis-elements in the HERV-PTN promoter that regulate the expression of the HERV-PTN fusion transcript. Furthermore, we identified several transcription factors by electrophoretic mobility shift assays that bind to these regulatory promoter elements of the PTN promoter.

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Introduction

Angiogenesis allows a solid tumor to grow beyond 2 mm^3 and provides the pathway for metastasis. The switch of tumor cells to the angiogenic phenotype involves a change in the equilibrium of positive and negative regulatory factors which control the proliferation of endothelial cells. One positive modulator of this process is **pleiotrophin (PTN)**, a secreted growth factor originally purified from the highly malignant breast cancer cell line MDA-MB231 (1). PTN depletion in these cells leads to a reduction of their tumor growth *in vivo* (2) and PTN overexpression in breast cancer MCF-7 cells results in their enhanced tumor growth *in vivo* correlated with increased vascular density (3). Taken together, the angiogenic growth factor PTN is discussed as one major factor for the malignant phenotype of human mammary carcinoma.

As we have been shown earlier, an endogenous retrovirus-like element (HERV-E.PTN) is located inside of the human PTN gene directly upstream of the first coding exon (6). Due to this insertion, a phylogenetically novel, internal promoter is generated which mediates the expression of functional HERV-PTN fusion transcripts in normal trophoblasts and in trophoblast-derived choriocarcinoma cells (4,5,6). Additionally, RT-PCR and in situ hybridization analysis from our lab, showed expression of HERV-PTN fusion transcripts also in some breast cancer cell lines and invasive mammary carcinoma specimens.

In this proposal we will investigate the hypothesis that utilization of the HERV-derived PTN promoter is one significant mechanism by which breast cancer cells increase their expression level of the angiogenic growth factor pleiotrophin, which potentially supports the transition from the avascular to the vascular stage. We will study the PTN expression pattern in invasive and noninvasive primary human mammary carcinomas and characterize the regulatory mechanism(s) responsible for the expression of HERV-PTN fusion transcripts in human breast cancer cells.

Proposal Body

In the approved Statement of Work three tasks were outlined.

Task 1. Expression status of PTN and HERV-PTN fusion transcripts in human mammary carcinoma (month 8 to 12).

Task 2. Characterization of the mechanism(s) of HERV-PTN fusion transcript expression in human breast cancer cell lines (month 1 to 30).

A. Transcriptional activity of the HERV-derived PTN promoter in breast cancer cell lines (month 1 to 18).

B. Identification of cis-elements in the HERV-derived PTN promoter (month 14 to 30).

C. Examine whether posttranscriptional regulatory mechanisms are involved in the HERV-PTN fusion transcript expression in human breast cancer cell lines (month 28 to 36).

Task 3. Effects of hormones and hormone antagonists on the HERV-PTN fusion transcript expression (month 32 to 42).

- The following report about our progress includes data showing progress concerning Task 2 (month 1-30).

Task 1: The goal of our studies regarding Task 1 was to study the expression of PTN and HERV-PTN fusion transcript in human mammary carcinoma.

Progress regarding Task 1 was documented in the first Annual Report (1 Sep 1999 – 31 Aug 2000).

Task 2: The focus of Task 2 is the analysis of the regulatory elements of the HERV-derived PTN promoter. Specifically we are interested in regulatory cis-elements of this promoter and their interaction with the transcription factors that bind to these elements.

Identification of novel regulatory elements of the HERV-PTN promoter.

In previous studies we showed that insertion of a human endogenous retrovirus like element (HERV) into the PTN gene generated a promoter that mediates the expression of functional HERV-PTN fusion transcripts in normal trophoblasts and in trophoblast-derived choriocarcinoma cells. We demonstrated using transient transfection experiments that the region +404/+631 is critical for transcriptional activation of this promoter (Fig. 1 top). Here we analyzed the contribution of various cis-elements in the +404/+631 region of this promoter by serial 3' deletions of the promoter starting at position +631. These deletion constructs were obtained by exonuclease digestion and are shown in Fig.1 (total of 19 deletion constructs: Fig. 1 bottom). The deletion constructs were cloned in front of a luciferase reporter gene and transiently transfected into JEG-3 and HeLa cells and analyzed by luciferase assays. As Fig. 2 shows, we could identify two regions (+550/+631 and +486/+529) which contained cis-activating elements (characterized through the loss of promoter activity after deletion) and one region (+529/+550) which contained a strong cis-repressor element (characterized through the strong activation of promoter activity after deletion). Since we have found relative low HERV-PTN promoter activity in HeLa cells and various breast cancer cell lines (data not shown and Fig. 5) we were especially interested in the region +529/+550, which seemed to harbor a strong

repressor element for this promoter. In order to identify the factor responsible for the repressive effect we next performed electrophoretic motility shift assays (EMSA's).

Identification of transcription factors that bind to the HERV-PTN promoter.

In order to identify transcription factors that bind to the repressive cis-element in the HERV-PTN promoter, we performed electrophoretic motility shift assays (EMSA's) with synthetically synthesized promoter fragments. First we tested various point mutations or double mutations of a +528/+551 promoter fragment for transcription factor binding by EMSA (data not shown). We identified three potential regions to which sequence specific factors were binding. By computational analysis using TESS (Transcription Element Search Software) we screened the transcription factor binding site databank 'Transfac' and found one potential AP1 site and one potential YY1 site. The third binding site (called M-factor binding site) resulted in no previously identified factor. In order to confirm these results we performed EMSA's with the +528/+551 promoter fragment and mutated AP1 and/or YY1 sites as well as EMSA-supershift experiments using antibodies directed against these factors. These experiments demonstrated that AP1 and YY1 bind to this HERV-PTN promoter fragment (Figs. 3 & 4). In order to analyze whether these factors have also a functional role for the promoter we next mutated the AP1 and YY1 sites and tested the mutated promoter fragments in transient transfection assays (see below).

Repression of the HERV-PTN promoter by the transcriptional repressor YY1 in human breast cancer cells.

In order to analyze the functional role of AP1 and YY1 in human breast cancer cells, we mutated the binding sites of these factors in the HERV-PTN promoter and analyzed the resulting promoter activity in transient transfection assays. As Fig. 5 demonstrates, while mutation of the AP1 binding site had little effect on the promoter activity, mutation of the YY1 binding site resulted in a strong increase of the HERV-PTN promoter activity in various breast cancer cells (we obtained the same results in JEG-3 and HeLa cells; data not shown). Thus, we identified in this study an important cis-repressor element in the HERV-PTN promoter, and showed that the transcriptional repressor YY1 has an important role for the expression of the HERV-PTN fusion transcript (and therefore for total PTN expression levels) in human breast cancer cells.

Task 3: The goals of our studies regarding Task 3 were to determine whether hormones and hormone antagonists have an influence on the expression of the HERV-PTN fusion transcript.

These goals will be, as planned, performed in years three and four of the funding period.

Key Research Accomplishments

- We generated 19 HERV-PTN promoter deletion constructs by serial exonuclease digestion of the HERV-PTN promoter fragment –600/+631.

- We identified two regions in the HERV-PTN promoter which contained cis-activating elements (+550/+631 and +486/+529) and one region which contained a strong cis-repressor element (+529/+550).

- We identified and characterized two transcription factors that bind to the repressor cis-element, AP1 and YY1.

- We demonstrated that YY1 represses the HERV-PTN promoter in human breast cancer cells.

Reportable Outcomes

- Generation of 19 HERV-PTN promoter deletion constructs.

- Generation of HERV-PTN promoter constructs with mutations for the transcription factors AP1 and YY1.

- Melih Babaoglu, Randa Melhem and Anke Schulte. Characterization of positive and negative regulatory elements in the HERV-PTN promoter region. Manuscript in preparation (the report contains many unpublished data and should therefore be protected and its distribution should be limited).

Conclusions

In this proposal we want to investigate whether utilization of the HERV-derived PTN promoter is one significant mechanism by which breast cancer cells increase the expression level of the angiogenic growth factor pleiotrophin. Specifically, we want to study PTN expression patterns in invasive and noninvasive primary human mammary carcinomas and characterize the regulatory mechanism(s) responsible for the expression of HERV-PTN fusion transcripts in human breast cancer cells.

During the first two years of this project we have identified important regulatory cis-elements in the HERV-PTN promoter that regulate the expression of the HERV-PTN fusion transcript. We identified in year two of the funding period, two regions in the HERV-PTN promoter which contain two cis-activating elements and one region which contains a strong cis-repressor element. Furthermore, we identified transcription factors that bind to these regulatory promoter elements of the PTN promoter. One of these transcription factors, YY1, represses strongly the transcription of the HERV-PTN fusion transcript in breast cancer cells. We anticipate that the results from the proposed

experiments, outlined in the approved Statement of Work, especially the discovery of the regulatory elements and transcription factors that bind to the HERV-PTN promoter, will give us valuable insights into the regulation of expression and the biological significance of the growth factor PTN in breast cancer.

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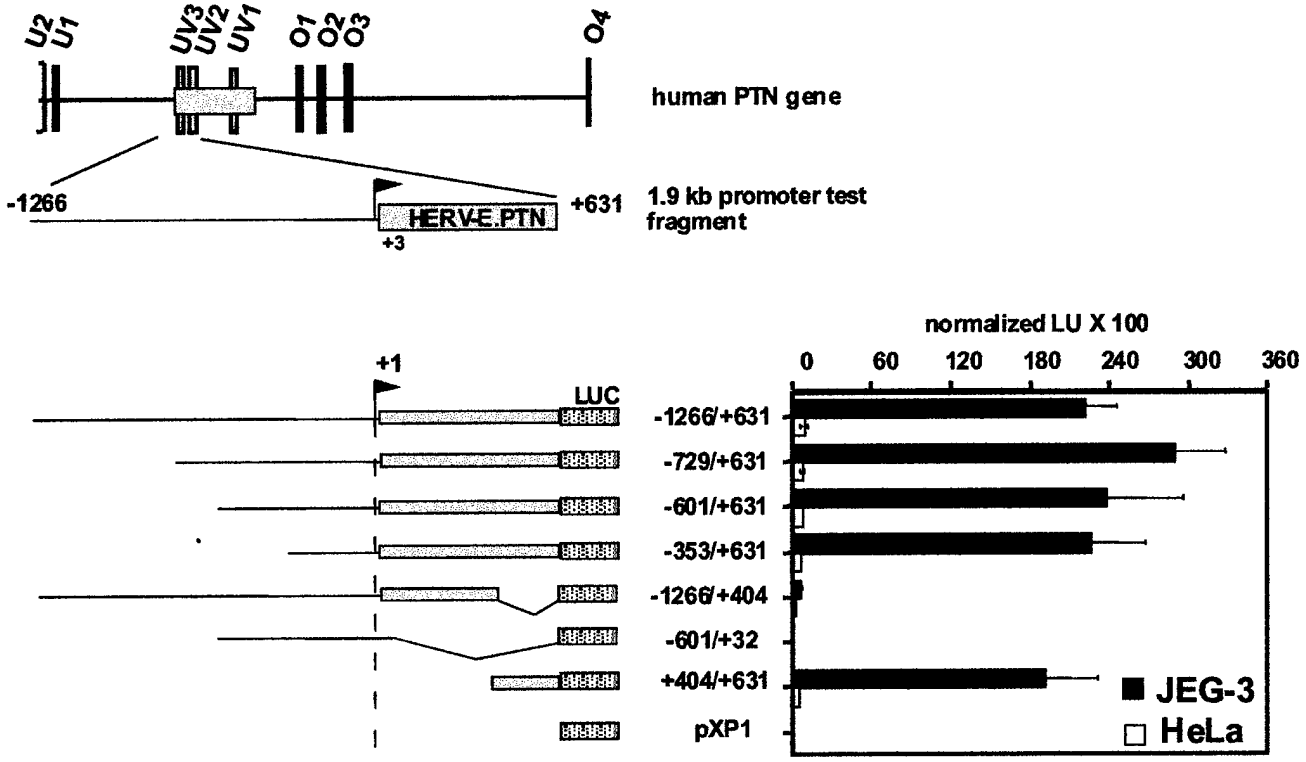
Appendix

A



B

Name	promoter-600
8B	631
11B	550
6D1	529
11D1	517
11D5	512
486	486
6E2	466
11C	462
6C	441
6D4	432
2C	396
9A	364
9C	341
1C	337
5C	307
7C	278
12A	217
10A	176
12C	172
3A	27

Fig.1: (A) HERV-PTN insertion transformes human PTN intronic sequence into promoter active sequence.

(B) List of the serial HERV-PTN promoter deletion fragments generated by exonucleasedigestion; 8B=wildtype promoter from -600 to +631; Numbers indicate 3'-primed end of the promoter

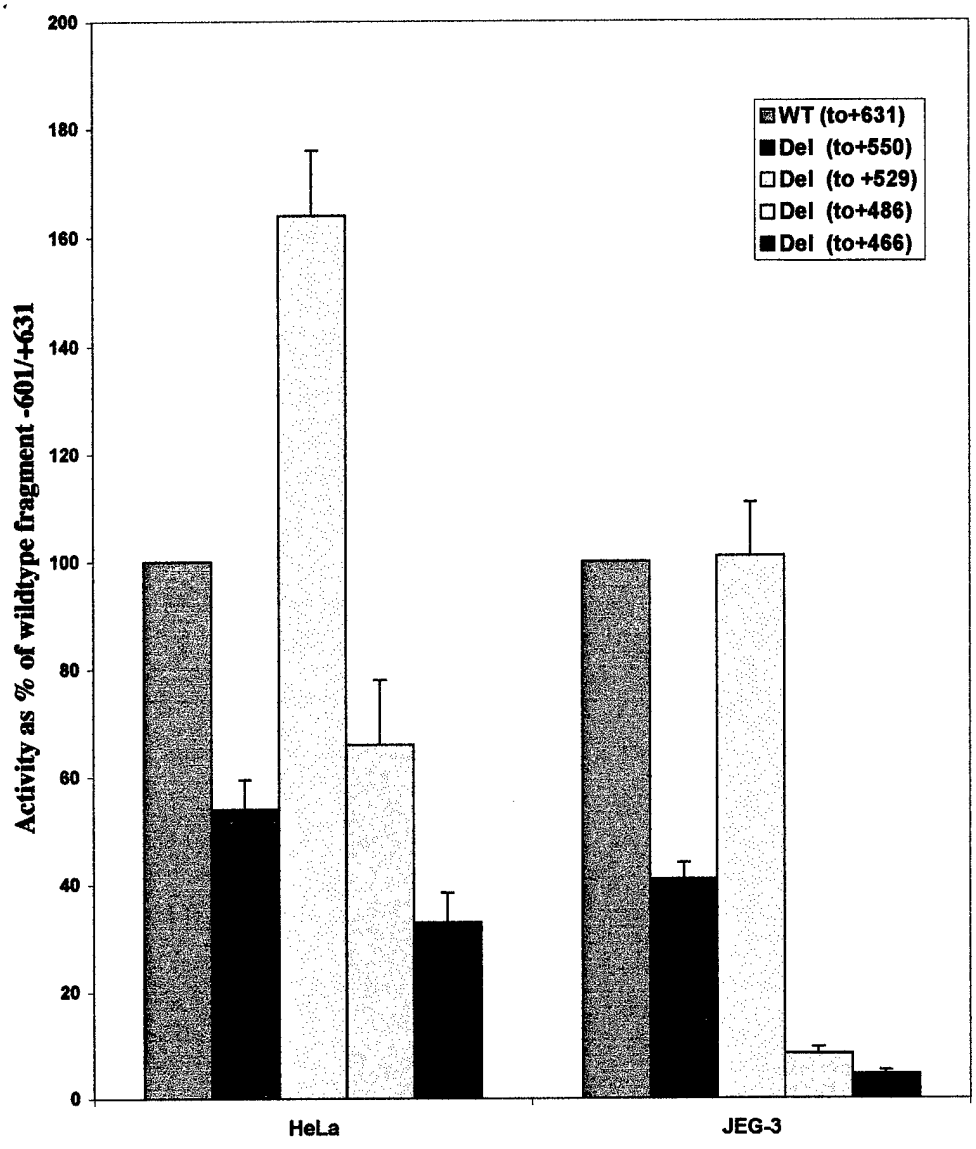


Fig.2: Deletion analysis of HERV-PTN promoter fragment -601/+631

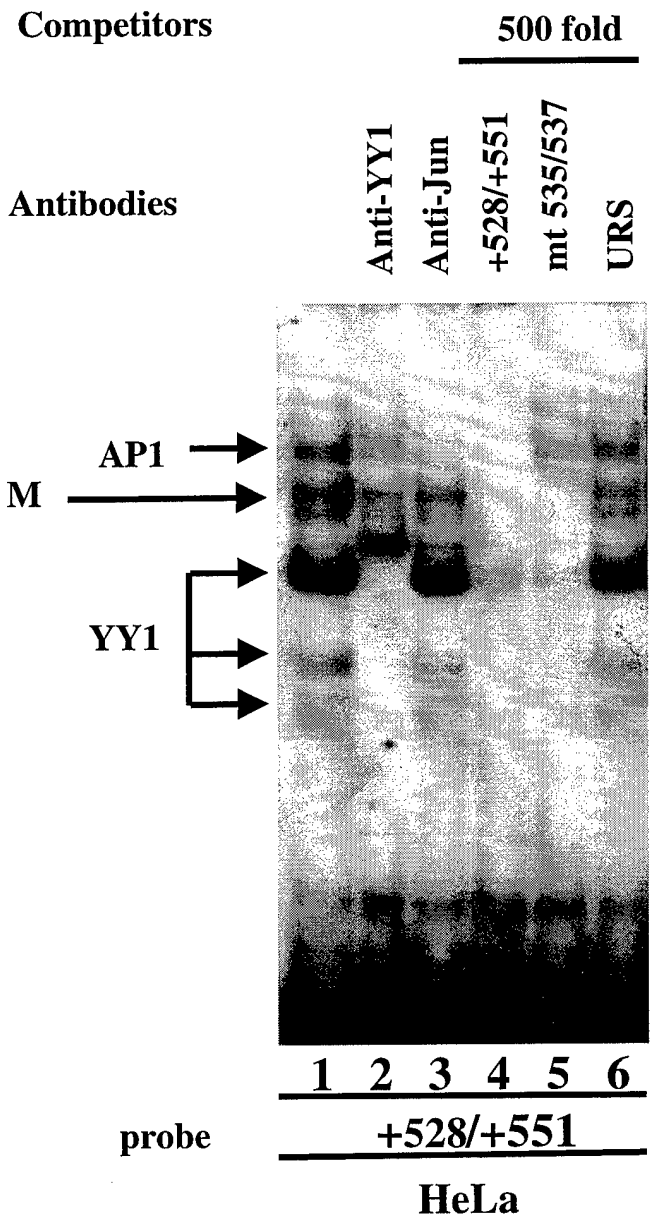


Fig. 3: Electrophoretic mobility shift assay (EMSA) of the HERV-PTN promoter fragment +528/+551 with HeLa extracts (URS: unrelated sequence).

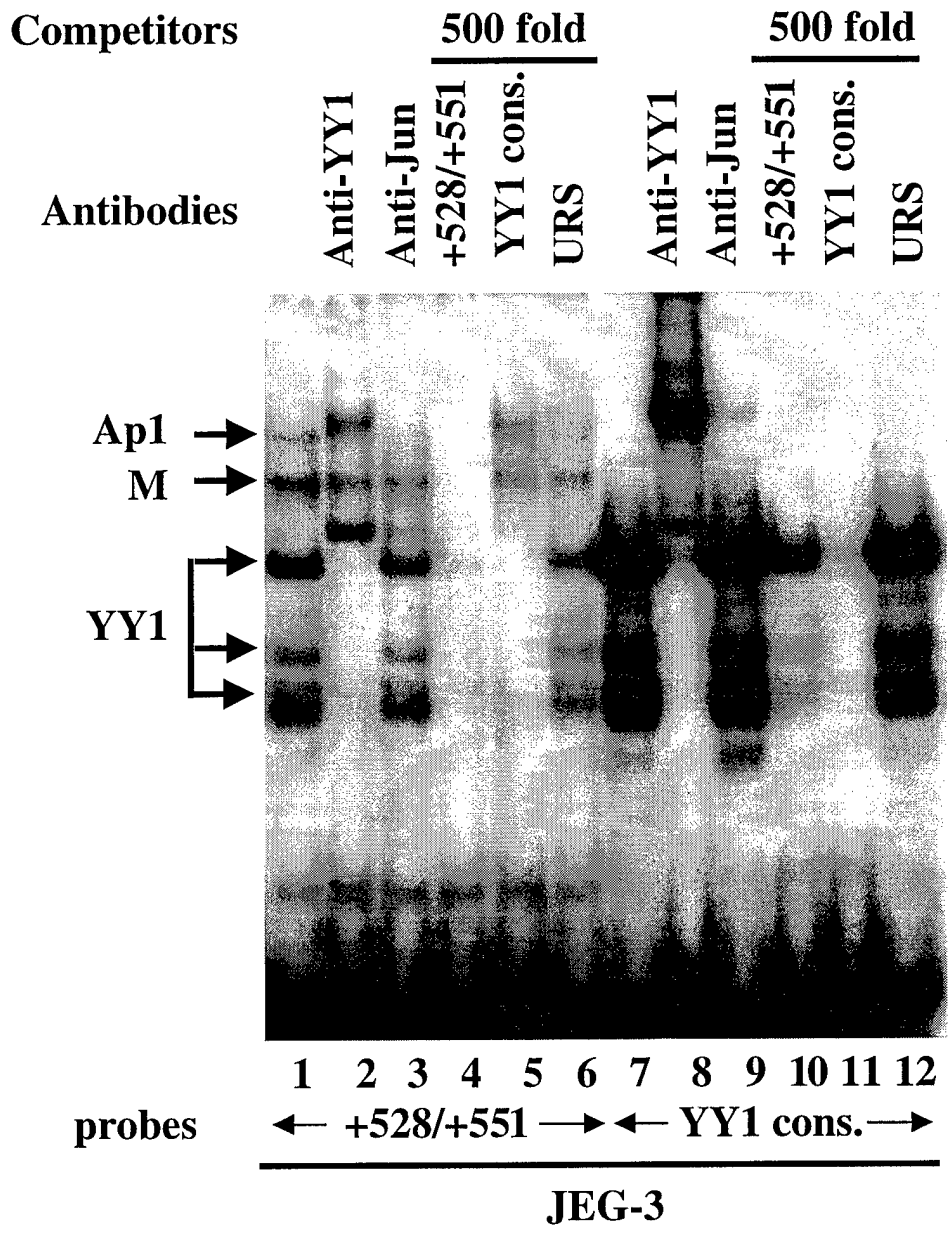


Fig. 4: Electrophoretic mobility shift assay (EMSA) of the HERV-PTN promoter fragment +528/+551 with JEG-3 extracts (URS: unrelated sequence).

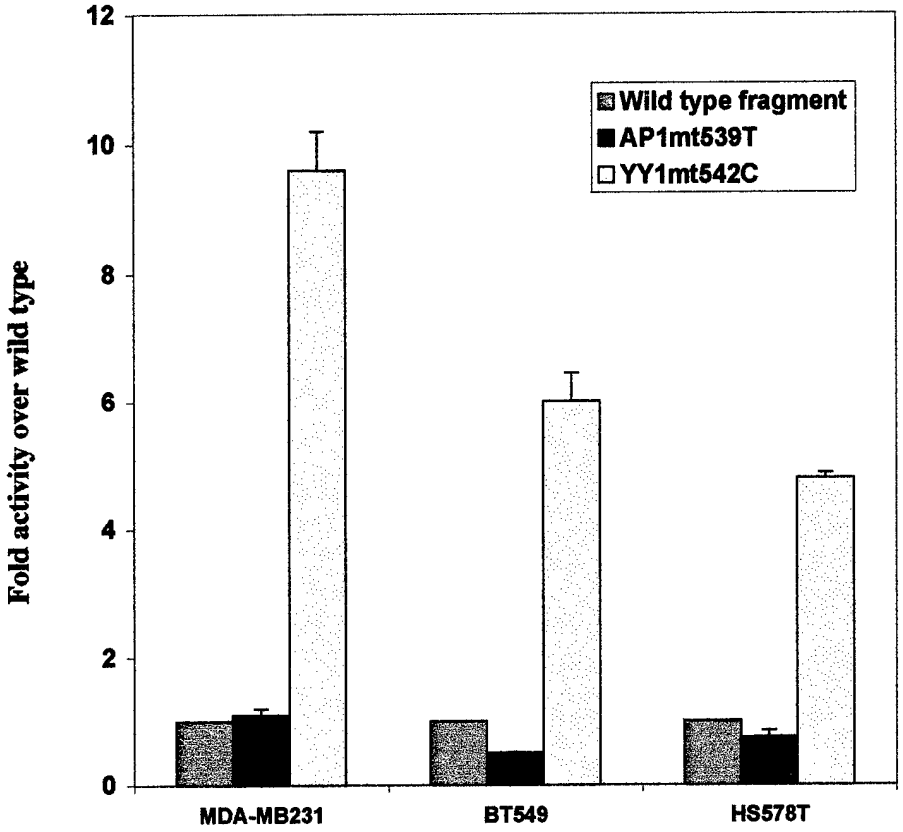


Fig. 5: Effect of AP1 and YY1 binding sites on the activity of HERV-PTN promoter in breast cancer cell lines