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13. ABSTRACT (Maximum 200 Words)
To identify promoter elements and transcription factors that contribute to enhanced expression of Vascular Endothelial Growth Factor (VEGF) in breast cancer, reporter constructs, encompassing nested deletions approximately every 100 base pairs (bp) of the wild type (wt)-promoter (-1175 to +50) were synthesized and screened for activity by transient transfection in human breast cancer and mom-mammary cells. In most cells, a drop of ≥ 40% in transcriptional activity was observed under normoxic conditions when sequences between positions -1175 and -1010 were deleted. Sequences downstream of the hypoxia-regulatory element (HRE) at position -971 modulate activity in a tissue- and cell type-specific manner: deletion of sequences between positions -900 and -790 reduces promoter activity another 30%, indicating differences in transcriptional regulation among tissues and within the same cell type. Electrophoretic mobility shift assays (EMSA) revealed that transcription factor Sp1, but not AP-2 or Egr-1, binds the basal promoter element upstream of the transcriptional start site between positions -85 and -50.

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INTRODUCTION

Expression of vascular endothelial growth factor (VEGF) is elevated in malignant cells, and clinical studies correlate increased levels of the protein with poor prognosis in breast cancer (1). Currently little is known about the transcriptional activation of the gene in this particular disease. We hypothesized that regulation of VEGF at the transcriptional level could be different from other types of cancer. Experiments were designed to identify elements in the wild-type (wt) VEGF promoter between positions –1200 and +50 that are responsible for increased transcription in breast cancer cells, and to characterize the transcription factors interacting with these elements. An understanding of how the gene is regulated in this disease could be used as a baseline for developing improved treatment regimens. We plan to synthesize novel expression vectors containing the VEGF promoter elements required for high-level expression in breast cancer cells. Therapeutic genes would thus be expressed specifically in malignant but less so in normal cells.

BODY

As outlined in the Statement of Work, year 2 included the identification and characterization of transcription factors that interact at functionally relevant VEGF promoter regions (Task 2) and the construction of core VEGF promoters that show high-level activity in breast cancer cells (Task 3). Part of the year was also spent completing Task 1, the identification of VEGF promoter elements, which are responsible for elevated transcriptional activity in breast cancer cells.

Task 1: Identify VEGF promoter elements responsible for the elevated transcriptional activation of the gene in breast cancer cells.

This task is now complete: 10 promoter constructs with progressive deletions approximately every 100 base pairs (bp) in 5' to 3' direction, starting at position –1175 upstream of the transcriptional start site were transfected into 4 human breast carcinoma cell lines (MCF-7, T-47D, MDA-MB231 and SK-BR3), 2 control cell lines (the human prostate cancer cell line PC-3 and the human kidney cell line 293) and 3 human lung carcinoma cell lines (A549, NCI-H292 and Calu-3).

Results are summarized in Figure 1, and the following findings emerge. It appears unlikely that breast cancer cells utilize unique cell type-specific promoter sequences for transcriptional activation of VEGF, and the overall expression patterns for different promoter constructs are

similar to those found in other cell lines. In general, deletion of the first 165 bp between positions –1175 and –1010 leads to a drop in activity of ≥ 40%. To our knowledge it has not been reported that sequences upstream of the hypoxia-regulatory element (HRE) at position –971 contribute to increased transcriptional activity (sequences upstream of position –1175 do not seem to contribute to increased transcription; data not shown). Further, sequences downstream of the HRE also modulate activity, albeit in a cell type-specific manner: deletions of sequences between positions –900 and –790 reduces activity another 30% in T-47D and MDA-MB231 cells, but not MCF-7 and SK-BR3 cells, indicating differences in transcriptional regulation among breast cancer cell lines. Interestingly, the deletion of sequences between positions –790 and –700 restores in part transcriptional activity in T-47D and MDA-MB231 cells, indicating the presence of a negative regulatory element within that region.

Analysis of all constructs was also conducted in cell lines that were kept under hypoxic conditions (1% O_2 for 16 hours) following transfection. Under these conditions the HRE is able to activate transcription several-fold (2). In our experimental setup we observed that this activation depends on cell density, which to our knowledge has not been reported (cell density affects VEGF expression in colon cancer cells under normoxia, *i.e.* 20% O_2 [3]). Under hypoxic conditions the HRE did not increase transcription in cells kept at ≤ 50% confluence. Overall activity of all constructs was not different from that in control cells kept at normoxia. However, if cells were approximately 90% confluent, increased activity for all constructs was found when compared to that in control cells (in our experimental setup we did not find differences in activity between normoxic high- and low-density conditions). This erratic behavior makes the collection of interpretable data difficult, and currently we have not finished collecting the data under hypoxic conditions.

However, the data from hypoxic cells collected so far suggest that with the exception of the HRE the same promoter regions are in use under both conditions. Figure 2 shows, as an example, the results obtained in MCF-7 cells. Overall the expression pattern is similar to that in normoxic cells, and only constructs that contain the HRE show between a 2.5- (p1.2) and a 5-fold (p1.01) higher activation. Activity between these two constructs still drops approximately 20% when sequences between positions –1175 and –1010 are removed, revealing the modulatory effect of this promoter region even under hypoxia. As expected, deletion of the HRE completely abolishes any transcriptional increases when compared to normoxia, and activity of all remaining constructs is essentially the same under both conditions. These results indicate that future analyses of interesting promoter sequences can be done mostly under normoxic conditions.

Task 2: Identify and characterize transcription factors that interact at functionally relevant VEGF promoter regions

This task is comprised of two goals: first, the identification of transcription factors that directly bind functionally important promoter elements using classical molecular techniques, and second, the characterization of transcription factor complexes that assemble at such sequences with matrix assisted laser desorption ionization/time of flight-mass spectroscopy (MALDI/TOF-MS).

To identify transcription factors that interact directly with regulatory important promoter regions we employed electrophoretic mobility shift assays (EMSA). The analysis of the promoter segment that constitutes the basal promoter upstream of the transcriptional start site between positions –85 and –50 was used to optimize the system. This fragment is one of the best-investigated promoter segments within the VEGF promoter, is responsible for low-level expression in all cell lines analyzed so far, and contains binding sites for several transcription factors (4-7). The results using nuclear extracts from two breast cancer cell lines are summarized in Figure 3.

Our results are similar to the ones previously published: as in other cell lines the transcription factor Sp1 is also the predominant protein interacting at this segment in breast cancer cells. Further, there are no differences in complex formation among the two breast cancer cell lines; all three Sp1 binding sites are most likely occupied by protein. Conditions that favor binding of the zinc-finger transcription factor Egr-1 did not change the binding pattern (data not shown). Also, binding of the cell type-specific transcription factor AP-2 is unlikely since MDA-MB231 cells do not express this protein (S. Baylin, Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, MD; personal communication).

EMSAs using the basal promoter element and nuclear extracts prepared from other cell lines were not performed as we anticipated similar results, with Sp1 being the dominant DNA-binding protein. Currently we are in the process of identifying potential transcription factor-binding motifs within the –1175/–1010 and –900/–790 segments and designing the appropriate oligonucleotides.

We have not started using MALDI/TOF-MS to analyze the composition of the complexes assembled at the basal promoter element, as optimization of the EMSA system to visualize specific complexes took longer than expected. We anticipate performing the first MS-experiments within the upcoming weeks.

Task 3: Construct core VEGF promoters with high-level activity in breast cancer cells

This task involves the design and construction of expression vectors that contain core VEGF promoter sequences. These core promoters will contain the functionally important promoter elements in various numbers and orientation.

This task has not started since the promoter regions within the –1175/–1010 and –900/ –790 segments are not sufficiently defined to make these experiments feasible. Preliminary data using the HRE to modify an existing expression vector, pGWiz (Gene Therapy Systems; San Diego, CA), containing a modified human cytomegalovirus promoter/enhancer revealed that inclusion of a single HRE copy alone does not convey any advantages. Measuring the activity in MCF-7 cells of such a chimera in comparison to a construct containing the wt-VEGF promoter did not show increased promoter activity under hypoxic conditions (data not shown). It was previously shown however, that five copies of the HRE could be selectively activated by hypoxia, making such a vector useful for tumor-selective gene therapy (8).

KEY RESEARCH ACCOMPLISHMENTS

- Analysis of 10 VEGF constructs with progressive promoter deletions approximately every 100 bps in 5' to 3' direction, starting at position –1175 upstream of the transcriptional initiation site were completed in 4 human breast cancer and 5 non-mammary control cell lines to identify sequences that contribute to increased transcriptional activity in breast cancer cells.
- Besides the HRE at position –971 and the basal promoter element between positions –85 and –50, sequences upstream and downstream of the HRE also contribute to transcriptional activation in a cell type- and tissue-specific manner: a drop of $\geq$40% in activity was observed when sequences between positions –1175 and –1010 were deleted. Within the same type of cells, activity could drop another 30% when sequences between positions –900 and –790 were removed.
- A potential negative regulatory element was also identified within the VEGF promoter: deletion of sequences between positions –790 and –700 restores in part transcriptional activity back, albeit not in all cell lines.
- EMSA with nuclear extracts from human breast cancer cells identified the transcription factor SP1, but not AP-2 or Egr-1, as the only protein binding directly to the basal promoter element upstream of the transcriptional start site.

REPORTABLE OUTCOMES

1. Bredow, S., Falgout, M.M., and Malkoski, S.P. Transcriptional regulation of VEGF in berast cancer cells. Proceedings of the American Association for Cancer Research *44*:995, 2003.

CONCLUSIONS

The results so far suggest it to be highly unlikely that promoter elements specific for breast cancer are involved in the elevated expression of VEGF in this disease. The analysis of the promoter revealed that, besides the HRE and basal promoter elements, which are involved in VEGF regulation in all cell types, there are at least two more activating sequences involved in transcriptional activation in most cells: an element located upstream of the HRE between positions –1175 and –1010 and an element located downstream of the HRE between –900 and –790. Interestingly, this element is not active in all breast cancer cells, indicating differences in regulation not only among different tissues but also within the same class of cancer cells. A more detailed analysis of these sequences will reveal the specific sequence motifs that modulate the gene's activation. Although it is beyond the scope of the present proposal, a detailed analysis of the potentially novel negatively regulatory element within the VEGF promoter between positions –790 and –700 also seems reasonable: elucidating the *cis*-regulatory sequences and transcription factor complexes that influence the expression of VEGF may identify novel regulatory pathways whose modulation could be used to decrease the progression of cancer growth.

These results also influence Task 3, the construction of gene therapy expression vectors. It seems most feasible now to design and test a minimized promoter that contains only the activating elements, before changes in number and orientation of such elements should be attempted. In comparison to the 1200 bp wt-promoter, a minimzed promoter also allows easier handling and manipulation during the testing for functionality of promoter sites. For one, the necessary mutations can be introduced during its construction. Second, since the activity of such a promoter will also be close to that of the wt-promoter, changes in activity are easier to observe. For example, were construct p0.1 to be used to test the functionality of the three Sp1-sites, changes would be small due to the inherent low activity of this construct. With the exception of the HRE, all of the activating sequences can further be tested under normoxic conditions. To resolve the existing experimental problems regarding the activation by hypoxia, we are currently testing whether to include metals such as Cobalt and Manganese in our experimental setup. These metals are known to mimic hypoxic-like conditions in cells (9), and we have shown in a

related project that Manganese induces the wt-VEGF promoter in lung cancer cells under low-density conditions.

Since analysis of the basal promoter element by EMSA did not reveal any new information about transcription factor identity and functionality, we mainly used this system to optimize the setup in order to allow future analysis of complex composition and characterization (e.g. post-translational modifications) by MALDI/TOF-MS at the newly identified, activating promoter elements. To detect tertiary interactions with co-regulators that might get lost during the migration through the gel, we will however, also employ a technique recently described by Nelson *et al.* (10). This modified EMSA uses oligonucleotides that are modified at their 5'-end with Acrydite to allow for covalent linkage to acrylamide. A binding reaction can thus be polymerized into an acrylamide matrix within the well of the precast gel. Proteins complexed to the Acrydite DNA are trapped and can be separated through electrophoretic migration of the unbound proteins, followed by elution from the gel to allow enzymatic digests for MALDI/TOF-MS peptide mapping.

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APPENDICES

1. Figures 1- 3
2. Copy of abstract published in the Proceedings of the American Association of Cancer Research.

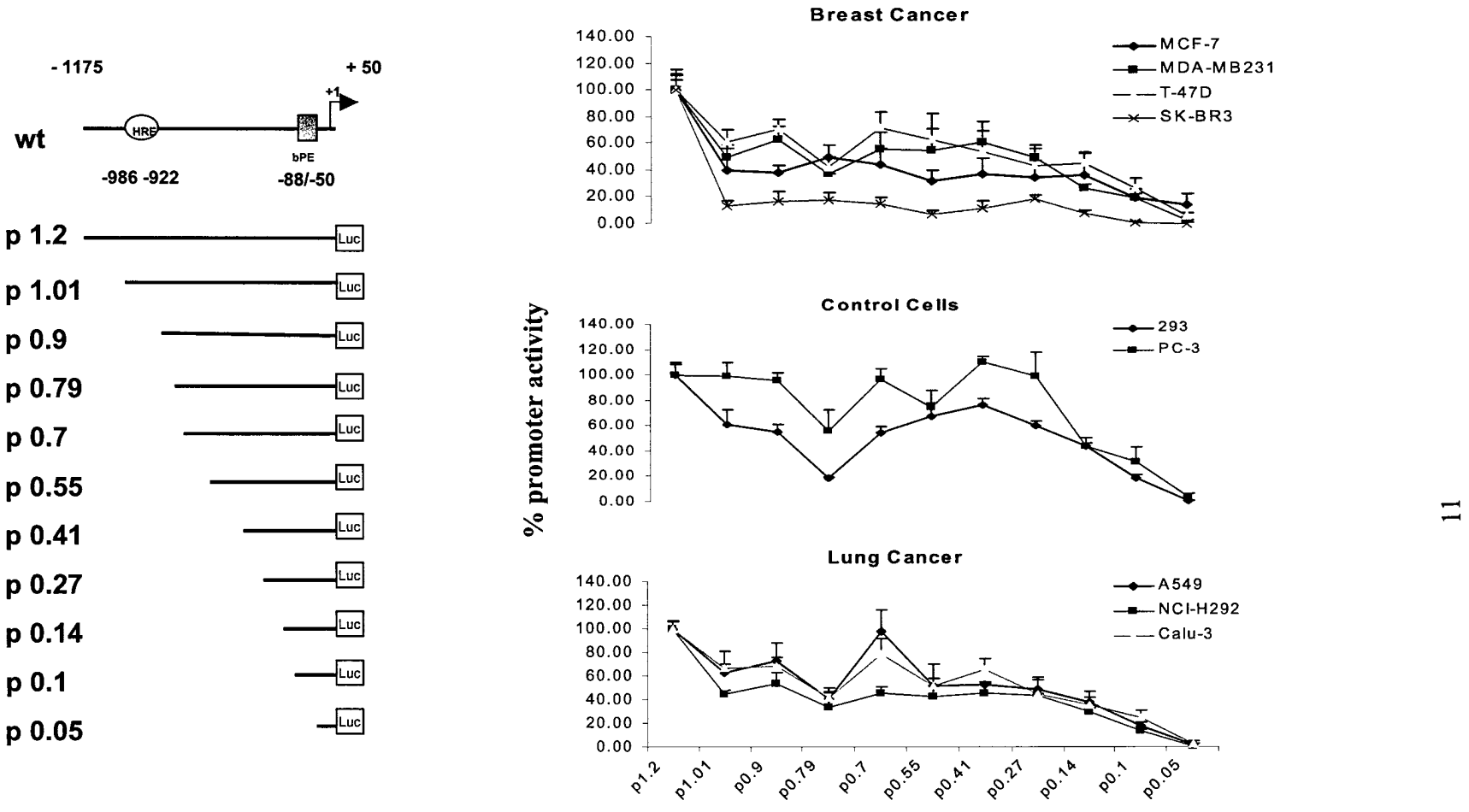

Figure 1: Expression of 5' nested VEGF gene promoter deletions in various cell lines following transient transfection. Constructs, their names and size in comparison to the wild type (wt)-promoter are shown on the left. Within the wt-promoter (-1175/+50) the positions of the hypoxia-regulatory element (HRE) and the basal promoter element (bPE) are indicated, the transcriptional start site is marked by an arrow. Transfections, using FuGENE 6 (Roche Molecular Biochemicals; Indinapolis, IN) and 0.05 pmol for each construct to keep promoter concentrations constant, were done in duplicats and repeated at least thrice for each cell line. Promoter activities were determined by the ratio of firefly luciferase (Luc) to that of co-transfected control *Renilla* luciferase and are presented as mean ± SD. Activity of construct p1.2, which contains the wt-promoter, was set to 100% and used to normalize the other constructs' activities. Due to variations among individual transfections, only changes of activity of ≥30% are considered significant among constructs.

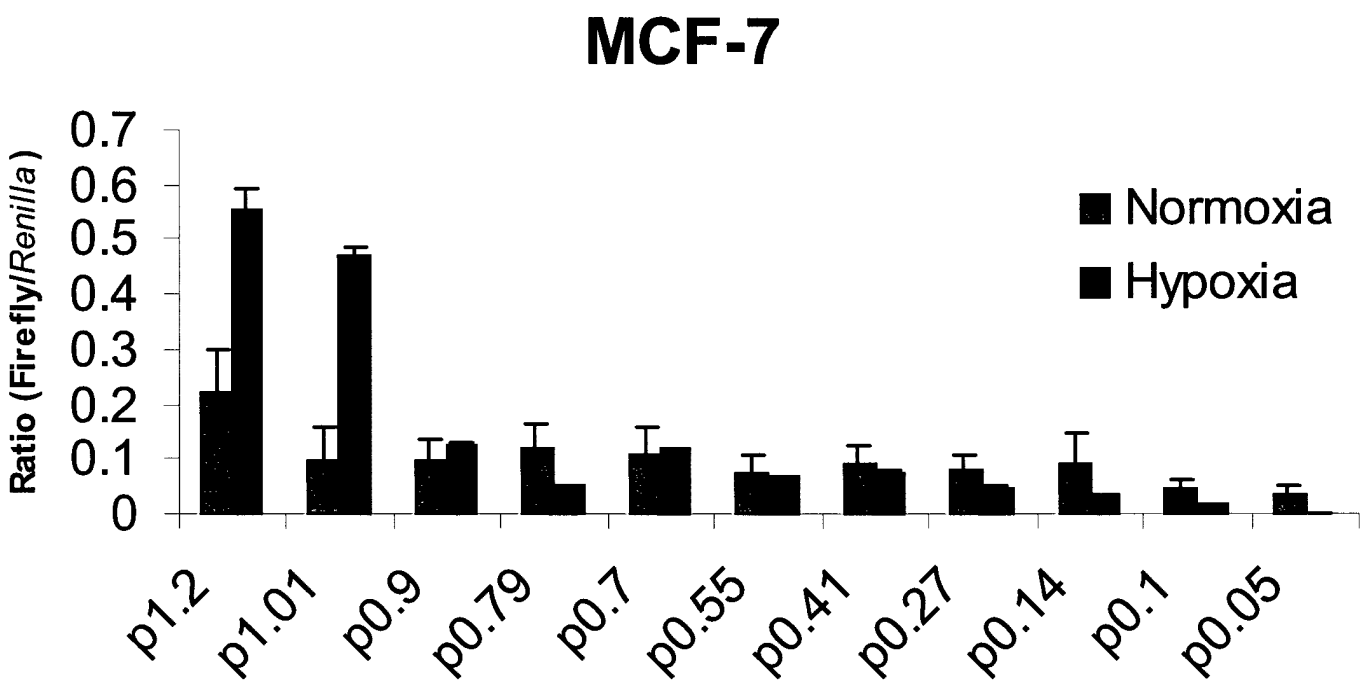

Figure 2: Comparison of promoter activity in MCF-7 breast cancer cells under normoxic and hypoxic conditions. Cells were at approximately 80% confluence when they were placed into a 3 gas incubator, set to 1% O_2, for 16 hours. Results show that the hypoxia-regulatory element is responsible for increased promoter activity under such conditions. Once removed, promoter activity is about the same as under normoxic conditions.

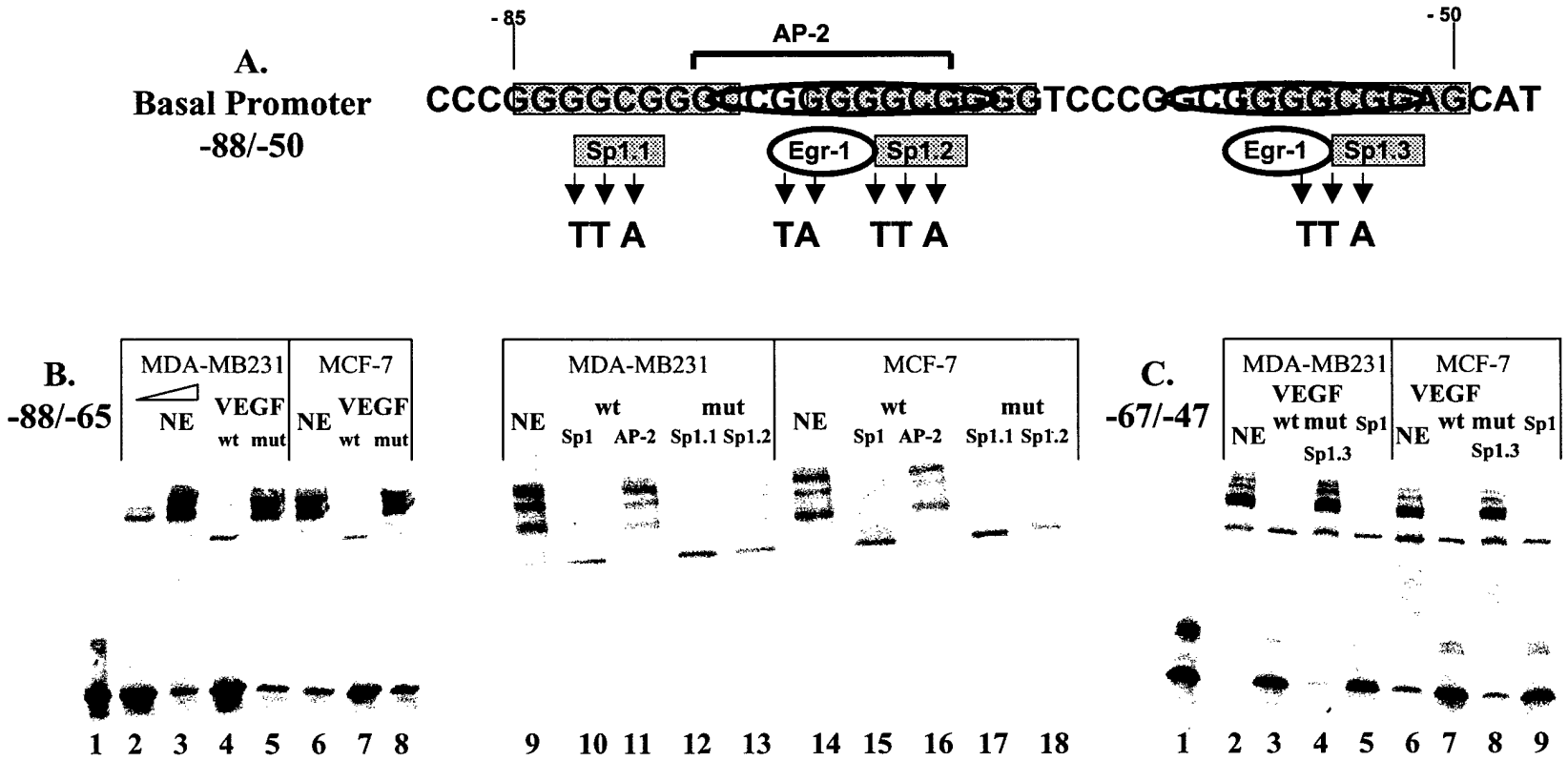

Figure 3: Analysis of transcription factor binding to the basal promoter element (bPE). A. Sequence of the bPE with bindings sites for transcription factors Sp1, AP-2, and Egr-1 between positions –85 and –50. Mutations that abolish binding of a specific transcription factor are also indicated. Two oligonucleotides were designed, encompassing positions –88/-65 (B.) and –67/-47 (C.). Nuclear extracts (NE) from two breast cancer cell lines, MDA-MB231 and MCF-7, were used as indicated. Lanes 1 respectively, represent free, unbound DNA. B. One (lane 2) and 3 µl (lanes 3-18) NE were used for the experiments. Complexes were resolved at 200V on 4-20% polyacrylamide gels, using 0.5xTBE as running buffer. Higher resolution revealed that 3 complexes form over the –88/-65 site (lanes 9-18). Complexes could not be formed in the presence of a 100fold excess of wt-(-88/-65) oligo (lanes 4 and 7), while the presence of an oligo, in which all binding sites were mutated (mut), had no effect (lanes 5 and 8). Competition with consensus oligos containing either the binding site for Sp-1 (lanes 10 and 15) or AP-2 (lanes 11 and 16) revealed that Sp1 but not AP-2 binds to the bPE. Both Sp1-sites, Sp1.1 and Sp1.2, seem functionable as competition with oligos, in which either site is mutated, are sufficient to abolish binding (lanes 12, 13, 17, 18). C. Incubation of 3 µl NE with the (–67/-47) oligo reveals Sp-1 binding : competition with the wt-oligo (lanes 2,5,7,9) abolishes complex formation while mutation of the Sp1-binding site has no effect (lanes 4 and 8).

Transcriptional Regulation of VEGF in Breast Cancer Cells

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Expression of Vascular Endothelial Growth Factor (VEGF) is often elevated in malignant cells, and clinical studies correlate increased levels of the protein with poor prognosis in many forms of cancer, including breast cancer. Currently little is known about the transcriptional activation of the gene in this disease.

To identify promoter elements that contribute to enhanced expression of VEGF in breast cancer, 10 reporter constructs, encompassing nested deletions approximately every 100 base pairs (bp) of the wild type (wt)-promoter (-1200 to +50) were synthesized and screened for activity by transient transfection in a panel of human breast cancer cells. Identification and characterization of transcription factors binding to the proximal promoter element upstream of the transcriptional start site between positions –88 and –65 was done by Electrophoretic Mobility Shift Assays (EMSA). This promoter segment contains binding sites for the transcription factors Sp1 and AP-2.

In all cell lines analyzed, a 40% drop in transcriptional activity to basal promoter activity was observed under normoxic conditions when sequences between positions –1175 and –1010, upstream of the hypoxia-regulatory element (HRE) were deleted. Interestingly, wt-like activity could be restored only in T-47D cells when sequences between positions –1175 and –420 were removed, indicating cell type-specific differences in transcriptional regulation in this disease. Further, in all cell lines all basal promoter activity was abolished when proximal promoter sequences between positions –100 and –50 were deleted. EMSA revealed two specific transcription factor complexes that bind between positions –88 and –65 in the proximal promoter. Competition experiments showed that the transcription factor Sp1, but not AP-2, was present in these complexes.

Elucidating the *cis*-regulating sequences that contribute to the overexpression of VEGF in breast cancer may identify novel regulatory pathways whose modulation could be used to decrease tumor growth.

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