Update on HER-2 as a target for cancer therapy
The ERBB2 promoter and its exploitation for cancer treatment

Helen C Hurst

ICRF Molecular Oncology Unit, Hammersmith Hospital, London, UK

Correspondence: Helen C Hurst, ICRF Molecular Oncology Unit, Hammersmith Hospital, London W12 0NN, UK. Tel: +44 020 8383 3972; fax: +44 020 8383 3258; e-mail: hurst@icrf.icnet.uk

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Abstract
Overexpression of the ERBB2 proto-oncogene is associated with amplification of the gene in breast cancer but increased activity of the promoter also plays a significant role. Members of two transcription factor families (AP-2 and Ets) show increased binding to the promoter in over-expressing cells. Consequently, strategies have been devised to target promoter activity, either through the DNA binding sites for these factors, or through another promoter sequence, a polypurine-polypyrimidine repeat structure. The promoter has also been exploited for its tumour-specific activity to direct the accumulation of cytotoxic compounds selectively within cancer cells. Our current understanding of the ERBB2 promoter is reviewed and the status of these therapeutic avenues is discussed.

Keywords: AP-2, ERBB2, Ets, gene therapy, promoter

Introduction
Over-expression of ERBB2 (HER-2/neu) is an adverse prognostic factor in human breast cancer, and may predict resistance to both chemotherapy and endocrine therapy (for review [1]). This over-expression was originally attributed solely to amplification of the ERBB2 gene (usually 2- to 10-fold), which frequently occurs both in tumours and in derived cell lines. It became clear, however, that over-expression can also be observed from a single copy gene [2]. Moreover, in all of the cell lines examined that exhibit gene amplification, an elevation in ERBB2 mRNA levels per gene copy was also observed [2]. This indicated that over-expression of the gene precedes and increases the likelihood of gene amplification, suggesting that further research into the transcriptional regulation of ERBB2 would be informative.

Consequently, a number of groups have used nuclear run-on assays to measure gene transcription rates, and these have shown an increase in transcription rate sufficient to account for the degree of over-expression in a number of breast tumour derived cell lines that over-express ERBB2 (for review [3]). Subsequent studies therefore sought to identify the gene sequences that are required to mediate this increase in transcription rate. This was largely done by examining the activity of reporter constructs that contain the major transcription initiation site plus various extents of the ERBB2 5′-flanking sequence, and hence comparing promoter activity in breast tumour lines with low and high levels of expression. The results of these experiments are summarized here, and attempts either to target promoter function or to exploit the differential activity of the ERBB2 promoter for use in genetic therapies are reviewed.

ERBB2 promoter structure and interacting factors
The human ERBB2 proximal promoter contains typical TATA and CCAAT boxes, at −22 to −26 bp and −71 to −75 bp, respectively (Fig. 1); it should be noted that the TATA box is not conserved in the rodent neu gene, making it difficult to compare studies across species, and therefore only data from the human gene are discussed here.

AP = activator protein; EBS = Ets-binding site.
Two regions of transcription initiation have been mapped within the ERBB2 promoter; one grouped around the major start site at +1, with minor starts centred at −69 (Fig. 1). Transcription initiation at these two sites appears to occur by two separate mechanisms, with the upstream start site being specified by and dependent upon an initiator-like element, whereas the downstream sites require the presence of the TATA box. In over-expressing cells it is the −69 initiation site that appears to be preferentially upregulated (for review [3,4]). An additional feature of the ERBB2 promoter is a 27 bp polyuridine (GGA)/polypurine (TCC) mirror repeat at −40 to −66 (Fig. 1). This sequence has been reported to overlap a putative matrix attachment region within the promoter, and it has the potential to form a distinct architectural conformation known as Hr-DNA, which is an internal triplex structure with a single-stranded D-loop [4].

ERBB2 promoter activity was initially demonstrated in reporter assays using sequences from −500 to +40 [5,6]. Subsequently, a whole range of reporter constructs were used by different laboratories containing up to 6.0 kb of ERBB2 5′-flanking sequence (for review [3]). The majority of those studies also compared reporter activity in breast cell lines with either high or low expression of ERBB2. Clear differential activity was observed, with sequences up to −300 being able to mediate the majority of this effect, whereas further 5′-flanking sequences had little positive influence on promoter activity. These findings tally well with separate studies mapping nuclear DNase I hypersensitive sites, which often indicate regions of regulatory importance within a gene. One site was mapped within the ERBB2 promoter, centred on the region of the CAAT and TATA boxes, and extending both upstream and downstream [7,8], although it is not possible to define the precise limits. No other hypersensitive sites were detected within 6 kb 5′ of +1, suggesting that much of this region may be inaccessible to trans-acting factors in vivo and that the only sequences of regulatory significance reside within the proximal promoter. One additional hypersensitive site upstream of −6.0 kb was observed [8]. Intriguingly, there has been a report that an additional ERBB2 promoter, associated with alternative 5′ exons, exists 12 kb upstream of the conventional start of the gene [9]. However, preliminary experiments in these laboratories have indicated that, although the region probably contains a functional promoter, it does not demonstrate differential activity between cells with low and high expression of ERBB2 (Brown N, unpublished data).

A number of transcription factors have been demonstrated to bind to the ERBB2 proximal promoter (for review [3,4]). However, only two of these, namely the AP-2 and Ets families of transcription factors (Fig. 1), have been shown to be both required for maximal promoter activity and associated with over-expression of the gene in breast cancer. The AP-2 binding site (GCTGCAGGC) at −213 to −221 was originally identified as a DNase I footprinted site that was protected by nuclear extracts from ERBB2 over-expressing cells (but not those with low expression), indicating differential DNA-binding activity of a nuclear factor, which was termed OB2-1. Mutation of the site to prevent binding resulted in impaired reporter activity in ERBB2 over-expressing cells [10]. OB2-1 was subsequently shown to be the developmentally regulated transcription factor AP-2 [11], which consists of a family of three highly homologous proteins, AP-2α, AP-2β and AP-2γ, all of which can activate the ERBB2 promoter [12]. Levels of AP-2α and AP-2γ proteins have been shown to correlate with ERBB2 over-expression in tumour derived cell lines and primary breast tumours [12,13].

The Ets binding site (EBS; GAGGAA), at −33 to −28, maps over a DNase I hypersensitivity site, the intensity of which is more marked in footprinting assays that use extracts from over-expressing cells [4,7]. Moreover, mutation of this site also impaired reporter activity [4,7,14]. It has been reported that binding of Ets factors to the EBS induces a severe bend in the DNA [4]. It is therefore likely that if the EBS is occupied then the TATA-binding protein will not be able to access the closely associated TATA box, making EBS binding an attractive explanation for the increased use of the −69 start site that is observed in over-expressing cells. At least 10 different Ets proteins...
have been found in breast cells at varying levels. Of those, only PEA3 has so far been shown to correlate in distribution with ERBB2 over-expression [15], but there have been conflicting reports as to whether this protein activates or represses the ERBB2 promoter [4,14].

**Targeting ERBB2 promoter function**

Although the precise molecular mechanisms that govern ERBB2 promoter activity in over-expressing cells and in those with low levels of expression are far from being totally defined, a number of strategies have been explored to limit promoter activity in over-expressing cells. The rationale is that it will be more efficient to reduce ERBB2 levels by preventing the transcription of 2–10 gene copies than trying to neutralize up to $10^6$ receptor molecules commonly found in over-expressing cells. The main strategies used to date fall into two groups: those targeting the AP-2 or EBS and those targeting the polypurine/polypyrimidine repeat structure.

The AP-2 and Ets sites have been targeted by either preventing the binding of the cognate binding protein (using sodium aurothiomalate for AP-2 [16] and DNA-binding polyamides for Ets factors [17]) or by creating fusion proteins between the appropriate DNA-binding domain and a transcriptional repressor [4], in all cases impairing promoter activity in functional assays. These approaches have only been used in vitro, but another strategy, based on the observation that over-expression of the Ets factor PEA3 reduced ERBB2 expression, has also been examined in preclinical trials. Breast and ovarian tumour lines with either low expression or over-expression of ERBB2 were grown as xenografts in nude mice and treated with daily injections of liposome-conjugated PEA3 expression plasmid. Significant reductions in tumour growth were observed in the ERBB2 over-expressing lines, with some mice surviving over a year, whereas growth of the tumours with low expression was unaffected [14].

The polypurine/polypyrimidine repeat provides an interesting target because of its ability to bind triplex-forming oligonucleotides. A number of papers have described successful triplex targeting, resulting in a reduction in Ets factor binding at the adjacent EBS (for review see [4]). Reduced promoter activity has also been reported in cells transduced with a retrovirus expressing an antigenic RNA targeted to the same sequence [18]. Perhaps the most novel approach, however, is the construction of six-fingered zinc finger proteins designed to bind 18 bp of the polypurine/polypyrimidine repeat in a genome-specific manner. Fusing a variety of repression domains to these synthetic DNA-binding proteins resulted, in one case, in total ablation of promoter activity in in vitro reporter assays [19].

None of the above strategies have yet proceeded to patient trials, but another approach has now entered clinical trials. Work on the rodent neu promoter revealed that coexpression of certain tumour-related proteins, namely adenovirus E1a, SV40 large T antigen, c-Myc or the retinoblastoma gene product Rb, could apparently downregulate promoter activity (for review [3]). The mechanism by which this occurs is unclear; it is certainly indirect and may not actually be related to expression of ERBB2 or neu. Nevertheless, experiments in animal models were promising enough to lead to a phase I trial in which cationic lipids were used to transfer an E1a expression plasmid into ERBB2-positive breast and ovarian tumour cells. The approach was found to be safe with no dose-limiting toxicity and showed some antitumour effects, notably increased apoptosis. A phase II trial in head and neck cancer patients, however, indicated that E1a is effective irrespective of the ERBB2 status of the tumour [1].

**Using differential ERBB2 promoter activity in genetic prodrug-activation therapy**

Genetic prodrug-activation therapy is designed to selectively express a non-mammalian metabolic ‘suicide’ gene in tumour cells such that a coadministered non-toxic prodrug will be converted into its toxic metabolite only within the tumour cells. In principle, this can be achieved by using a differentially active promoter such as ERBB2 to drive expression of the suicide gene. Preclinical trials using the *Escherichia coli* cytosine deaminase gene, which can convert 5-fluorocytosine to 5-fluorouracil, showed that cell toxicity could be limited to ERBB2 over-expressing cells using this strategy [20]. This led to a phase I clinical trial in 12 patients with skin nodule metastases of their primary breast tumours. The ERBB2/cytosine deaminase expression plasmid was injected directly into a nodule, with an adjacent nodule receiving a control injection of vector DNA alone. The approach was shown to be safe, and significant levels of suicide gene expression specifically restricted to the ERBB2 positive tumour cells were observed, thus demonstrating the selectivity of the approach [21].

**Conclusion**

Better delivery vehicles are required if advances in gene therapy approaches are to continue. Adenoviral vectors, a common choice for cancer gene therapy, were found to suppress the differential activity of the ERBB2 promoter, but this may be overcome by using genetic tricks such as the use of so-called insulator elements [22]. In addition, although the ERBB2 promoter is selectively active, it is not very potent, and so combinations with additional regulatory elements either from other genes such as MUC1 [23] or potentially using recently discovered ERBB2 intronic enhancers [8] may be used to increase expression of the delivered gene. The nature of this gene itself is open to wide variation, and strategies to increase tumour cell sensitivity to chemotherapy or radiotherapy by delivering appropriate genes are also being investigated [24].
In terms of transcriptional therapy, it is unlikely that systemic drugs that are specific either for AP-2 or Ets factors will be developed in the short term. Therefore, the strategies that are most likely to enter the clinic are use of gene therapy vectors to deliver transcriptional repressor molecules to ERBB2 over-expressing cells. Possible candidates include PEA3 [14], the synthetic polydactyl zinc finger proteins [19], AP-2 or Ets-repressor fusion proteins [4], or possibly a recently identified factor termed ZONAB. This is Y-box transcription factor that binds a site just downstream of +1 (Fig. 1) and represses ERBB2 promoter activity in a cell-density-dependent manner [25]. These approaches also need further evaluation, however; the relative potency of these different molecules must be directly compared, followed by preclinical testing in order to determine how safe and effective they may be in vivo. An additional consideration is the likelihood of an immune response, particularly to the synthetic molecules; this may actually prove to be an advantage in tumour eradication, although the lack of an immunocompetent model for breast cancer may make this difficult to evaluate.

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