The EMBO Journal vol.12 no.6 pp.2369-2375, 1993

A novel transcription factor, OB2-1, is required for overexpression of the proto-oncogene c-*erb*B-2 in mammary tumour lines

Donal P.Hollywood and Helen C.Hurst[1]

Gene Transcription Laboratory, ICRF Oncology Group, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK

[1]Corresponding author

Communicated by M.Fried

The c-*erb*B-2 receptor tyrosine kinase proto-oncogene product is overexpressed in 20-30% of breast carcinomas and this has been shown to correlate with poor prognosis. Previous analysis of tumour-derived lines has demonstrated that although the c-*erb*B-2 gene is often amplified, overexpression can occur from a single-copy gene. Moreover, whether or not the gene is amplified, overexpressing cells produce 6- to 8-fold more mRNA per gene copy than low-expressing cells. In this paper, we examine the possible mechanisms causing this deregulation of c-*erb*B-2 mRNA accumulation. Nuclear run-on studies indicated that the extra mRNA accumulation was due to increased transcription of the gene in overexpressing cells. Promoter analyses using c-*erb*B-2 5′ flanking sequences linked to CAT showed that the promoter is more active in overexpressing cells. Coupling promoter deletion functional studies with footprinting experiments, using nuclear extracts derived from both low and overexpressing cells, allowed the identification of a DNA-binding protein, OB2-1, which is considerably more abundant in a range of overexpressing lines. We discuss the possible role of OB2-1 in c-*erb*B-2 overexpression in breast tumour lines.

Key words: c-*erb*B-2 overexpression/transcription factor OB2-1

Introduction

The human proto-oncogene, c-*erb*B-2, is normally expressed at low levels in a variety of human adult epithelial cells (Press *et al.*, 1990). In contrast, the protein is overexpressed in 20-30% of carcinomas of the breast (King *et al.*, 1985), stomach (Kameda *et al.*, 1990), ovary (Slamon *et al.*, 1989) and pancreas (Williams *et al.*, 1991). This phenomenon has been most intensively studied in breast carcinoma where high levels of c-*erb*B-2 expression have been shown to correlate with poor prognosis (Slamon *et al.*, 1987), and to predict a poorer response to chemotherapy (Gusterson *et al.*, 1993) and endocrine therapy (Wright *et al.*, 1992).

Initial studies on primary mammary carcinoma samples have shown that overexpression of the c-*erb*B-2 protein is often accompanied by amplification (but not rearrangement) of the gene (King *et al.*, 1985). However, significant overexpression of c-*erb*B-2 protein can also be achieved from a single-copy gene (King *et al.*, 1989; Yamada *et al.*, 1989; Iglehart *et al.*, 1990; Kury *et al.*, 1990; Parkes *et al.*, 1990). Consequently, amplification of the c-*erb*B-2 gene alone cannot account for the levels of protein observed in these carcinomas. This was further borne out by studies, initially by Kraus *et al.* (1987) and followed up by other groups (Hynes *et al.*, 1989), comparing the gene copy number and the c-*erb*B-2 mRNA and protein levels in a number of mammary cell lines. The cells examined included immortalized, non-tumorigenic cells, as well as tumour lines exhibiting either low, normal levels of c-*erb*B-2 (low expressors) or others with high levels of c-*erb*B-2 protein (overexpressors). In general, the levels of the 4.5 kb c-*erb*B-2 mRNA and the 185 kDa protein in these cell lines were concordant. However, the levels of c-*erb*B-2 mRNA were seen to be 6- to 8-fold higher per gene copy in overexpressing tumour cells compared to low-expressing cells. This implies that whether or not the c-*erb*B-2 gene is amplified in the overexpressing cells, there is some deregulation in the process of c-*erb*B-2 mRNA accumulation as compared to the low-expressing cell lines.

Sequences flanking the 5′ side of the c-*erb*B-2 gene have been cloned and sequenced to −1500 (Hudson *et al.*, 1990) and transcription initiation has been shown to occur at one major site (designated +1) which lies 178 bp upstream of the translation initiation codon (Ishii *et al.*, 1987; Tal *et al.*, 1987). A TATA box at −25 and a CAAT box at −75 have been identified upstream of this transcription start site. Furthermore, the 5′ flanking sequences have been linked to the CAT reporter gene and shown to possess promoter activity in a variety of cell lines (Ishii *et al.*, 1987; Tal *et al.*, 1987; Hudson *et al.*, 1990). However, analyses of c-*erb*B-2 promoter activity have not so far been performed in mammary epithelial lines.

In this paper, we describe our investigations into the mechanism of c-*erb*B-2 overexpression in human mammary lines with a single-copy gene. By combining results from nuclear run-on assays and mRNA half-life studies, we conclude that the gene is more actively transcribed in overexpressing cells. Furthermore, results of transfection assays with c-*erb*B-2 promoter-CAT fusion plasmids and from DNase footprinting of the promoter have led to the identification of a DNA-binding factor which is more abundant in overexpressing cells, where it is apparently required for the increased promoter activity observed in these cells. We have called this factor OB2-1 for overexpression of c-*erb*B-2, factor 1.

Results

Characterizing mammary lines for c-erbB-2 expression

Initially, in order to establish that we could duplicate previous findings, we collected a panel of mammary cell lines including c-*erb*B-2 low and overexpressing tumour lines and immortalized, non-tumorigenic lines. Total RNA and genomic DNA were prepared from each line for analysis on Northern and Southern blots (see Materials and methods).

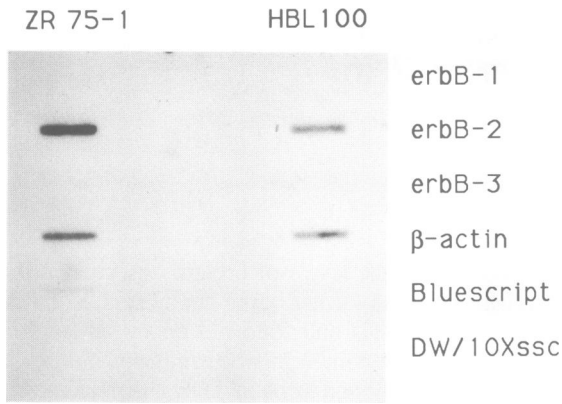

Fig. 1. Nuclear run-on assay. Nylon membranes carrying linearized plasmids were prepared as described in Materials and methods. Separate membranes were hybridized with equal counts from a run-on assay using either HBL100 (low-expressor) or ZR75-1 (high-expressor) nuclei. Filters were then washed and autoradiographed together as shown, and densitometrically scanned.

The results are summarized in Table I in terms of c-*erb*B-2 mRNA level per gene copy. As reported by others (Kraus *et al.*, 1987; Hynes *et al.*, 1989), if the level of mRNA per gene is set at 1 for low-expressing cells, then overexpressing cells show an average 6- to 8-fold greater accumulation of c-*erb*B-2 mRNA per gene copy, whether or not the gene is amplified. Many of these breast-derived lines are very slow growing. Therefore, to simplify our further studies on the mechanism behind this phenomenon, we have concentrated on the fastest-growing single-copy-overexpressing line ZR75-1 compared with the low-expressing tumour line T47D or the immortalized, non-tumorigenic line HBL100.

The c-erbB-2 gene is more actively transcribed in overexpressing cells

One explanation for increased accumulation of c-*erb*B-2 mRNA would be a slower rate of degradation of this RNA in overexpressing cells. To examine this question, we have looked at c-*erb*B-2 mRNA levels in cells treated for various times with actinomycin D to inhibit transcription. We found that the c-*erb*B-2 mRNA was very stable in both ZR75-1 and HBL100 cells, with an approximate half-life of 8–12 h (data not shown). Consequently, differences in half-life do not apparently account for the differences in c-*erb*B-2 mRNA accumulation observed in these cells.

Another mechanism that would account for increased c-*erb*B-2 expression in overexpressing cells would be if the gene were more actively transcribed in these cells. Nuclear run-on experiments (see Materials and methods) were performed on nuclei isolated from HBL100 and ZR75-1 cells. The amount of radiolabelled c-*erb*B-2 and β-actin RNA obtained in each incubation was determined by hybridization with an excess of cDNA immobilized onto a nylon filter, as shown in Figure 1. Densitometric scanning of the autoradiograph showed that the c-*erb*B-2 signal from the ZR75-1 cells is 3.8-fold greater than that obtained for HBL100 cells, if the two actin signals are normalized. As the amount of labelled RNA produced during a nuclear run-on experiment is proprotional to the number of polymerases already engaged on the gene at the time of harvest of the nuclei, this result indicates that an increased rate of c-*erb*B-2 transcription is contributing to the increased accumulation of mRNA in overexpressing lines.

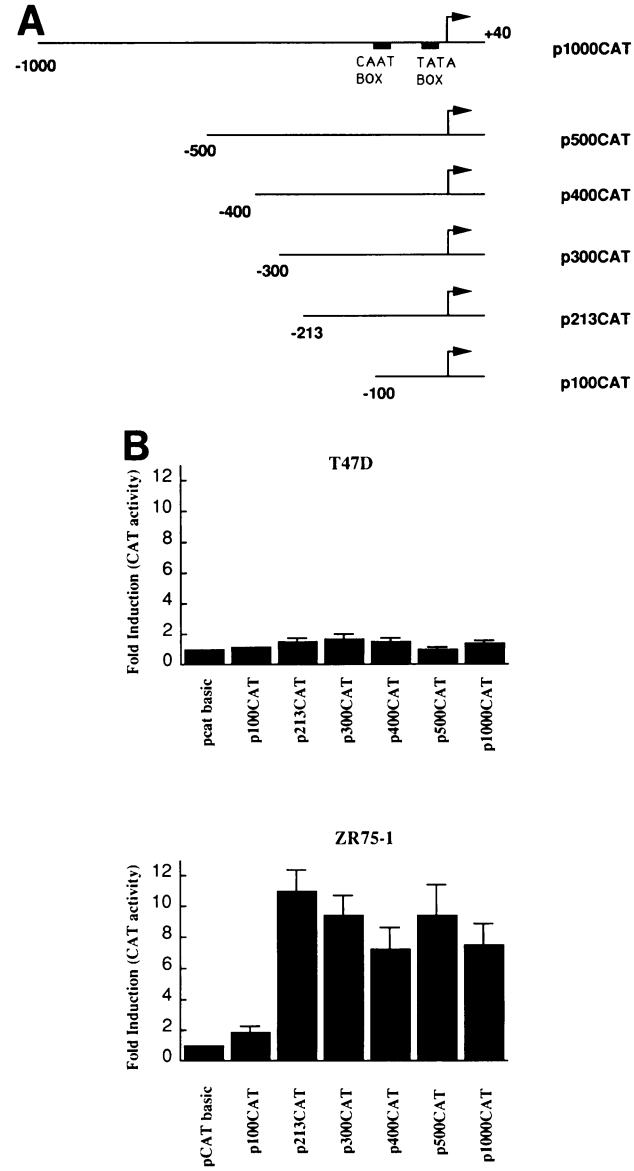

Fig. 2. Deletion analysis of c-*erb*B-2 5′ flanking regions. (**A**) c-*erb*B-2–CAT constructs. The regions of c-*erb*B-2 5′ flanking DNA used in each construct are indicated. The 3′ end point of all constructs was the *Sma*I site at +40 which was fused to the CAT coding sequences (see Materials and methods). (**B**) CAT assays with deletion mutants. For each set of transfections, the CAT activity from the promoterless pCATbasic plasmid is set at 1 and CAT activities from other constructs are expressed relative to this value (see Materials and methods). The average of at least five experiments is shown. Parallel assays using pSV2CAT confirmed the ability of each cell line to produce high CAT activity (data not shown). The bottom panel shows results with the high-expressing ZR75-1 line; the top panel shows results with the low-expressing T47D line.

The c-erbB-2 promoter is more active in overexpressing cells

The results from the nuclear run-on experiments indicated that the rate of transcription of the c-*erb*B-2 gene was higher than that in the overexpressing cells. This implies that the c-*erb*B-2 promoter may be more active, thus permitting more efficient transcription initiation in these cells. To test this hypothesis, we constructed a series of fusion genes consisting of c-*erb*B-2 5′ flanking sequences linked to the CAT reporter gene (see Materials and methods). These chimeric genes are illustrated in Figure 2A: the 3′ end of the c-*erb*B-2 sequences

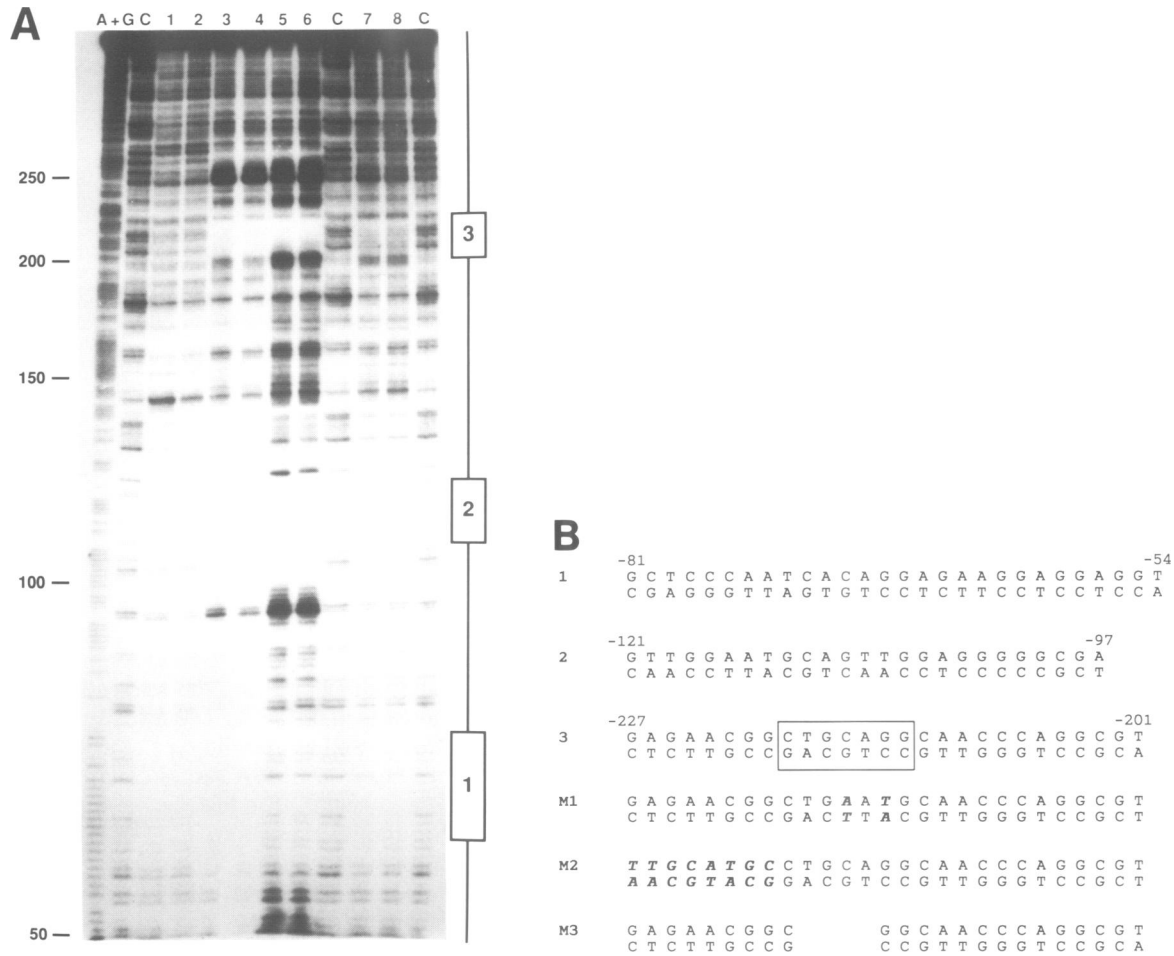

Fig. 3. DNase I footprinting of the c-*erb*B-2 promoter. (**A**) A fragment representing sequences −7 to −500 of c-*erb*B-2 5′ flanking DNA ^{32}P labelled on the non-coding strand at −7 to generate a probe for DNase I footprinting. Lane A+G shows a Maxam and Gilbert sequencing ladder generated from this probe. Lanes C represent control incubations of the probe alone with DNase I. Lanes 1−8 represent digestion of the probe with DNase I subsequent to incubation with breast cell nuclear extracts; the odd-numbered tracks had 120 μg and the even-numbered had 150 μg of crude nuclear protein, respectively. The origin of cell extracts was as follows: lanes 1 and 2: HBL100; lanes 3 and 4: ZR75-1; lanes 5 and 6: BT483; lanes 7 and 8: T47D. Numbers on the right indicate base pairs upstream of the +1 start site. Boxes on the left delineate the three footprinting regions detected by this probe. (**B**) The sequences delineated by each footprint in (A) are shown. Footprint 1 centres at −65, footprint 2 is termed the −100 region footprint and footprint 3 is termed the −213 footprint in the text. The box in footprint 3 shows the core binding sequence defined by methylation protection studies (see the text) and includes the *Pst*I site at −213. M1 represents footprint 3 with two point mutations (in bold) within the core sequence. M2 represents the sequences spanning the 5′ deletion end point in p213CAT; in light type are the c-*erb*B-2 sequences common to footprint 3 and in bold type are plasmid sequences immediately 5′ of the *Pst*I site. M3 represents the sequence around −213 within the mutant p500(KO)CAT (see Figure 6). The gap shows where 4 bp have been deleted relative to the wild-type sequence.

in all the constructs was +40 and the 5′ end was variable with the longest possessing >1 kb of sequences 5′ of the transcription start site. Similar constructs have previously been used by others to demonstrate c-*erb*B-2 promoter activity (Ishii *et al.*, 1987; Tal *et al.*, 1987).

The promoter constructs were transfected into mammary cell lines using the calcium phosphate technique. The CAT activity observed was compared to the levels obtained in parallel transfections with the parent, promoterless pCAT-basic plasmid to give a measure of promoter activity. All of the transfection experiments were internally controlled by the inclusion of a β-galactosidase expression plasmid (see Materials and methods). Results from several transfection experiments into high- and low-c-*erb*B-2-expressing mammary lines are shown in Figure 2B. The longest construct, p1000CAT, had similar activity to pCATbasic in T47D cells, but 6-fold greater activity in the overexpressing ZR75-1 line. Thus, the c-*erb*B-2 5′ flanking sequences do appear to be more active at promoting transcription initiation in overexpressing cells. Deletion analysis of the 5′ flanking sequences showed that all the constructs tested had little or no activity in low-expressing cells. However, in ZR75-1 cells, there was a marked reduction in CAT activity on deletion from −213 to −100 (compare the activities of p213CAT and p100CAT in the bottom panel in Figure 2B). This result indicated that an important transcriptional activating element lies between these two deletion end points.

Overexpressing cells contain an additional DNA-binding activity

To complement the promoter function analyses described above, we undertook to map nuclear factor binding sites within the c-*erb*B-2 5′ flanking sequences by DNase footprinting (see Materials and methods). Nuclear extracts were prepared from low and overexpressing mammary lines, and an example of footprinting c-*erb*B-2 sequences between −7 and −500 is shown in Figure 3A. Extracts from all the cell lines consistently produced two weak footprints: one lay

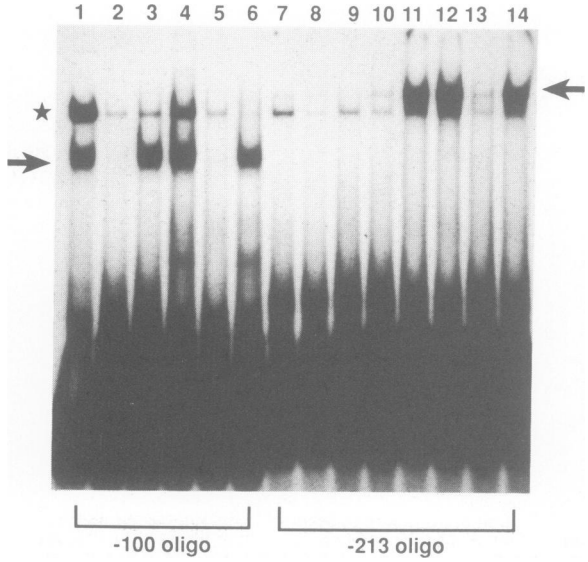

Fig. 4. EMSA assays with breast cell nuclear extracts. Specific binding of factors to the −100 and −213 ds oligonucleotides. **Lanes 1–6** have 0.1 ng/track ^{32}P-labelled −100 oligo probe and **lanes 7–14** have 0.1 ng/track ^{32}P-labelled −213 oligo probe. Lanes 1–3 and 7–10 contain 2.5 μg/track HBL100 crude nuclear extract. Lanes 4–6 and 11–14 contain 2.5 μg/track ZR75-1 crude nuclear extract. All lanes contain 1 μg poly(dA)/poly(dT) non-specific competitor and unlabelled specific competitor ds oligos as follows: lanes 1, 4, 7 and 11: none; lanes 2, 5, 8 and 12: 10 ng of −100; lanes 3, 6, 9 and 13: 10 ng of −213; lanes 10 and 14: 10 ng mutant, M1. The asterisk indicates a non-specific complex formed on all probes. The left-hand arrow indicates the specific complex on the −100 probe, while the right-hand arrow indicates the specific complex on the −213 probe.

in the region of the CAAT box at −75 (footprint 1, Figure 3A) and the other in the −100 region (footprint 2, Figure 3A). Nuclear extracts derived from the over-expressing breast tumour lines (lanes 3–6, Figure 3A) produced an additional strong footprint over the *Pst*I site at −213 (footprint 3, Figure 3A). The sequences covered by each of these footprints are shown in Figure 3B. Both footprints 2 and 3 fall within the most active region of the promoter identified in the deletion analyses described above.

The proteins binding to footprints 2 and 3 were investigated in further detail by electromobility shift (EMSA) assays. Double-stranded oligonucleotide probes representing each of the binding sites (see Figure 3B) were incubated with mammary cell nuclear extracts and analysed on non-denaturing gels. As shown in Figure 4, a distinct complex formed on each probe (arrowed) which could be competed only by the cognate binding site (Figure 4, lanes 1–6 and 11–13). DNA methylation protection assays were also performed (data not shown) and indicated that the core binding sequence of the −213 factor was CTGCAGG. This was further confirmed using a mutant oligo M1 (see Figure 3B) which incorporates two point mutations within this core binding motif and which failed to compete for factor binding in EMSA assays (Figure 4, lane 14).

The *Pst*I site at −213 was used to generate the p213CAT deletion which retains full promoter activity in the over-expressing cells (Figure 2B). The finding that the binding site for footprint 3 mapped directly over this site raises the question, therefore, as to whether p213CAT is still capable of binding the footprint 3 factor which thus contributes to the observed promoter activity. However, p213CAT retains

Table I. c-*erb*B-2 expression levels in mammary lines

Cell line	Gene copy number	Relative mRNA level[a]	mRNA/gene copy
HBL100[b]	1	1	1
MTSV 1.7[b]	1	1	1
BT20	1	1	1
T47D	1	1	1
MDA MB 231	1	1	1
MDA MB 436	1	1	1
MDA MB 175 VII	1	6–8	6–8
ZR75-1	1	4–6	4–6
BT 483	1	6–8	6–8
MDA MB 453	2	12–16	6–8
MDA MB 361	2–4	32[c]	8–16[c]
SKBR3	6–8	>50	6–8
BT474	4–8	64[c]	8–16[c]

[a]Expression relative to HBL100 cells.
[b]Immortalized non-tumorigenic lines.
[c]Derived from Kraus *et al.* (1987).

the core binding sequence CTGCAGG and, therefore, should allow binding of this factor. We have confirmed this using an oligonucleotide comprising the sequence spanning the deletion end point in p213CAT (Figure 3B, sequence M2). M2 competed as well as the wild-type sequence for binding of the −213 factor in EMSA assays (data not shown) and we therefore conclude that this factor will also bind to p213CAT in transfection assays.

The EMSA assays in Figure 4 showed that while the −100 region specific binding activity was present in all of the mammary cell extracts, only extracts from lines which overexpress c-*erb*B-2 contain the −213 specific activity (compare lanes 7–10 and 11–14 in Figure 4). We extended our analysis further by preparing nuclear extracts from a range of mammary cell lines (Table I) and performing further EMSA assays. In Figure 5, the bottom panel shows that all of the extracts contained the ubiquitous −100 region activity which also served as a check on the quality of these extracts. However, as shown in the top panel of Figure 5, only overexpressing lines contained significant levels of the −213 binding activity (compare lanes 1–6 with lanes 7–12).

OB2-1 binding is essential for maximal c-erbB-2 promoter activity in overexpressing cells

Both the −213 and −100 region binding activities lie within the portion of the c-*erb*B-2 promoter that is required for maximal activity in overexpressing cells (Figures 2B and 3A). However, from Figure 5 it is clear that the −100 activity is constant in all mammary-derived cell lines, while the −213 binding protein is only present at significant levels in overexpressing cells. Consequently, the presence of the −213 activity would apparently account most easily for the greater functional activity of the c-*erb*B-2 promoter in overexpressing cells. To test this hypothesis, we made a mutant within the −213 binding site of p500CAT such that the central 4 bp of the *Pst*I site which covers the core binding sequence were deleted to give p500(KO)CAT (see Materials and methods). This mutation (shown as M3 in Figure 3B) is more radical than the non-binding M1 mutation and would not be expected to bind to −213 factor. We have confirmed this by using oligos to the M3 sequence in competition assays; M3 is not able to compete for factor binding to the wild-type sequence. Moreover, when the M3 sequence was

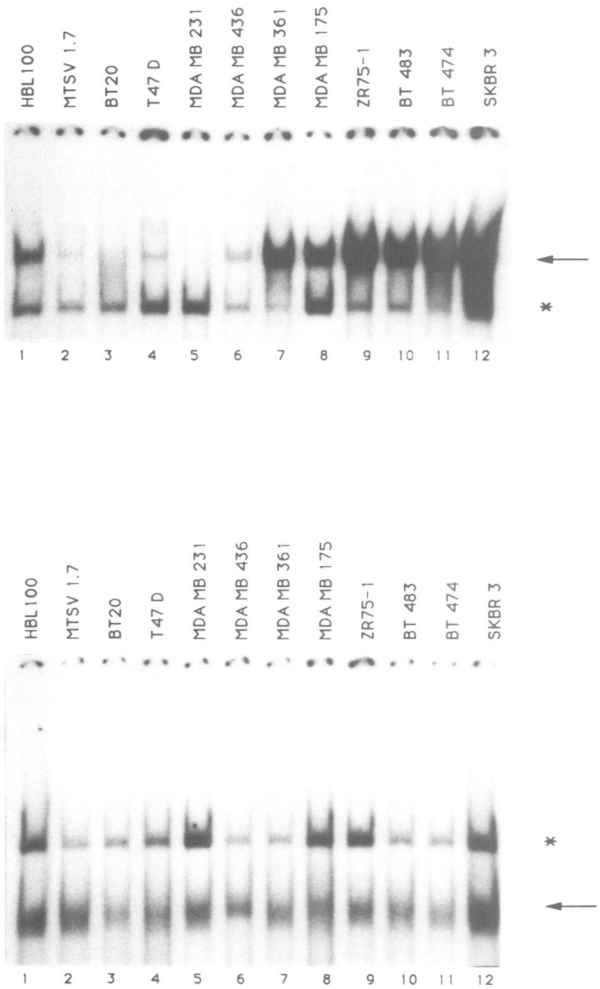

Fig. 5. Analysis of several breast lines for factor binding to the −100 and −213 oligonucleotides. The **top panel** shows factor binding to the −213 oligo and the **bottom panel** shows factor binding to the −100 oligo using the same nuclear extracts. All tracks contain 2−3 μg protein. Lanes 1−6 are extracts from low expressors of c-*erb*B-2 and lanes 7−12 are extracts from overexpressing cell lines (see Table I). In each panel, the arrow delineates the specific complex and the asterisk marks the non-specific complex (compare Figure 4).

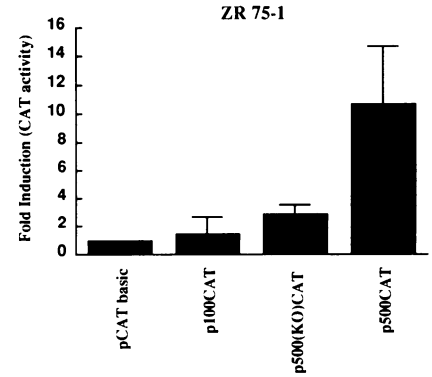

Fig. 6. CAT assays with the mutant, p500(KO)CAT. Short-term transfection assays into ZR75-1 cells were performed as described for Figure 2B. The CAT activity for each plasmid is expressed relative to the activity of the promoterless pCATbasic plasmid.

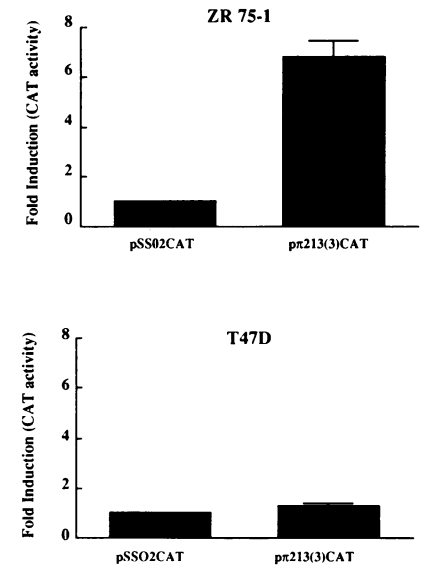

Fig. 7. CAT assays using heterologous promoter constructs. Short-term transfection assays into ZR75-1 and T47D cells were performed as described for Figure 2B. The CAT activity for the pπ213(3)CAT plasmid is expressed relative to the activity of the pSS0.2CAT plasmid, which achieved ~40% of the activity of pSV2CAT in each of the cell lines transfected.

used as a probe, we did not observe any complex formation with crude nuclear extracts (data not shown).

To test the functional significance of the M3 mutation, the activity of p500(KO)CAT was compared in transfection studies in ZR75-1 cells to the wild-type p500CAT, as shown in Figure 6. Clearly, the 4 bp deletion at −213 within p500(KO)CAT severely reduced the activity of the c-*erb*B-2 promoter to virtually basal levels. This implies that the major functional element within the c-*erb*B-2 promoter in overexpressing cells lies over the *Pst*I site at −213 and corresponds with the binding of a factor whose abundance is greatly increased in mammary cell lines which overexpress c-*erb*B-2.

As an additional demonstration of the functional importance of the −213 factor, we have also performed transfection assays with a construct containing several copies of its binding site upstream of a heterologous promoter. We have previously described the use of the reporter plasmid pSS0.2CAT which contains the basal promoter of the human glutathione transferase π gene (GST-π; Cowell *et al.*, 1992). We constructed a derivative of this plasmid, pπ213(3)CAT (see Materials and methods), which contained three copies of the footprint 3 sequence (see Figure 3B) upstream of the basal GST-π promoter. After transfection into the two mammary cell lines, the basal construct was surprisingly active as compared to our control plasmids. Nevertheless, pπ213(3)CAT showed a 6-fold increase in activity in the overexpressing line, ZR75-1, but no change in activity relative to pSS0.2CAT in the low-expressing line T47D (see Figure 7). Consequentely, taking together the results from several different promoter activity assays and the distribution of the −213 binding activity, we propose that the action of this factor on the c-*erb*B-2 promoter contributes to the accumulation of c-*erb*B-2 mRNA in overexpressing breast lines. We have therefore called this factor OB2-1 for overexpression of c-*erb*B-2, factor 1.

Discussion

In this paper, we set out to investigate the mechanisms causing the overaccumulation of c-*erb*B-2 mRNA observed

in many mammary tumour lines and in 20–30% of tumours. Using nuclear run-on assays and mRNA half-life studies in cell lines with a single-copy gene, we have established that the gene is more actively transcribed in cells overproducing c-*erb*B-2 mRNA. Transfection studies using the CAT gene fused to c-*erb*B-2 5′ flanking DNA have further shown that the promoter is more active in overexpressing cells. From results presented in this paper, we conclude that this increased promoter activity is mediated by a positively acting DNA-binding transcription factor, OB2-1, that is apparently more abundant in overexpressing cells. The importance of OB2-1 to the overexpression of c-*erb*B-2 mRNA in breast tumour-derived lines has been shown in several ways. First, the activity of the c-*erb*B-2 promoter in overexpressing cells is largely dependent on an intact OB2-1 binding site, as mutation of this site in a wild-type background reduced the activity of the promoter to near basal levels. Second, we have shown that the addition of OB2-1 binding sites to a heterologous promoter can increase the activity of that promoter in overexpressing cells, but not in low-expressing cells, thus underlining the functional importance of OB2-1 in the former cell type. Finally, when a large number of breast cell lines were examined for their OB2-1 content, the binding activity of the factor was found to correlate very closely with the known c-*erb*B-2 mRNA level in the same cell line (compare Figure 5 and Table I). Thus, non-tumorigenic and low-expressing tumour lines have little or no OB2-1-binding activity, while all lines that overexpress c-*erb*B-2, including those with amplified gene copies such as SKBR3 and BT474 cells (lanes 11 and 12, Figure 5), exhibit high levels of OB2-1 in EMSA assays. We therefore propose that the 6- to 8-fold increased accumulation of c-*erb*B-2 mRNA observed in overexpressing breast tumour cell lines is due to greater c-*erb*B-2 promoter activity in these cells mediated by a greater abundance of OB2-1.

This work also represents the first characterization of the human c-*erb*B-2 promoter in terms of interacting nuclear factors present in human mammary-derived cell lines. Some studies have been published looking at factors present in rodent fibroblasts which interact with the promoter of the rat and mouse homologue genes, *neu* (Suen and Hung, 1990; Yan and Hung, 1991; White and Hung, 1992). It is also interesting that the *neu* promoter contains an element capable of mediating transcriptional repression by the retinoblastoma gene product (Yu *et al.*, 1992). However, this *cis*-acting element is not conserved within the human c-*erb*B-2 promoter (Ishii *et al.*, 1987; Tal *et al.*, 1987). Moreover, we cannot find any similarity between the fibroblast factors and those we have identified in mammary extracts. The rodent genes lack the TATA box present in the c-*erb*B-2 promoter and consequently their transcription may be regulated in a quite distinct manner. Nonetheless, the binding sites for our footprints 1 and 2 are largely conserved in the rat promoter. In contrast, the binding site for OB2-1 is completely absent from the rodent genes.

We have also compared the OB2-1 binding site to sequences identified as nuclear factor-binding sites within other promoters. There is a close homology to a sequence found in some human keratin gene promoters which binds a factor thought to mediate keratinocyte-specific expression of these genes (Leask *et al.*, 1990). It is unclear at this stage if the two factors are identical; potentially, they may be members of a family of related proteins. Certainly, we have not detected OB2-1 in HeLa cells (where the keratinocyte factor has been found; Leask *et al.*, 1990) or indeed in other commonly cultured cells such as Hep G2, although a more exhaustive evaluation of the cell type distribution of OB2-1 is in progress.

There are several possibilities as to how mammary cells which overexpress c-*erb*B-2 acquired OB2-1. Obviously, the OB2-1 protein may also be overexpressed in these cells. However, another possibility is that the levels of the factor are actually very similar in all breast lines, but that the specific binding activity is elevated in overexpressing cells. This could be achieved if the DNA-binding activity of OB2-1 were normally regulated, perhaps through protein phosphorylation. The more active form might then be found constitutively within overexpressing cells as a consequence of changes occurring in regulatory pathways during progression to the transformed state. Intriguingly, preliminary experiments using extracts treated with phosphatase have indicated that OB2-1 is a phosphoprotein and that dephosphorylation results in a pronounced reduction in DNA-binding activity. To address these questions, we are proceeding to clone the gene for OB2-1 and raise antibodies to the protein. This will allow us to determine if increased OB2-1 activity also correlates with c-*erb*B-2 overexpression in primary tumour samples and also enable us to evaluate OB2-1 as a potential therapeutic target.

Materials and methods

Cell lines

Human mammary carcinoma cells and the non-tumorigenic HBL100 line were obtained from the ATCC and cultured in DMEM plus 10% fetal calf serum plus supplements, where indicated by the supplier. The MTSV1.7 line was obtained from Dr Joyce Taylor-Papadimitriou and cultured according to Bartek *et al.* (1991). The ZR75-1 cell line was obtained from Dr Malcolm Parker and cultured in RPMI 2% with 10% fetal calf serum.

Northern and Southern blotting and RNA half-life studies

Total RNA and genomic DNA were obtained from subconfluent cultured cells according to standard protocols (Maniatis *et al.*, 1989). For half-life studies, actinomycin D was added to 10 μg/ml of culture medium for various times up to 24 h. Inhibition of transcription was verified by pulsing with [α-^{32}P]UTP in parallel incubations. RNA samples (10 μg/track) were run on formaldehyde gels and blotted to a nylon membrane. DNA samples (10 μg) were digested with *Eco*RI, and run on agarose gels and blotted to a nylon membrane. Both types of blot were probed and washed under standard stringent conditions (Ausubel *et al.*, 1987) using the insert from a c-*erb*B-2 cDNA clone (Yamamoto *et al.*, 1986) as the probe. All blots were subsequently reprobed with the insert from a β-actin cDNA probe (Cleveland *et al.*, 1980) to control for loading. Slot blots with 2-fold serial dilutions of RNA were also used to help quantitate the level of c-*erb*B-2 overexpression.

Nuclear run-on assays

Nuclei were prepared from subconfluent cultured cells, and run-on assays and hybridizations were performed as described by Roberts and Bentley (1992). Nylon membranes carrying 5 μg/track of denatured dsDNA were prepared using a dot-blot manifold (Ausubel *et al.*, 1987). The *erb*B1 (Ullrich *et al.*, 1984) and *erb*B3 (Lemoine *et al.*, 1992) tracks contained whole, linearized plasmids. The *erb*B2 and actin tracks used DNA inserts prepared from the clones described above.

Generation of CAT reporter plasmids

Sequences flanking the 5′ side of the c-*erb*B-2 were cloned using PCR technology from human (HBL100) genomic DNA. All PCR-generated sequences were double-strand sequenced to ensure that they conformed in every respect to published c-*erb*B-2 5′ flanking sequence (Tal *et al.*, 1987; Hudson *et al.*, 1990). Sequences from +40 to −500 were obtained using 30 bases oligonucleotides to the sequences +40 to +10 and −497 to −467. Sequences from −500 to −1067 were obtained using 30b oligonucleotides

to the sequences −467 to −497 and −1067 to −1037. Subsequently, a fragment representing sequences −1067 to +40 of the c-*erb*B-2 5′ flanking region was generated by ligating the two pieces of amplified DNA at the *Sma*I site at −495. This piece was subcloned into the polylinker of the promoterless reporter plasmid pCATbasic (Promega) to generate p1000CAT. Further plasmids were constructed with 5′ deletions, either by using restriction enzymes [*Sma*I at −495 (p500CAT); *Pst*I at −213] or further PCR reactions using the +40 to +10 oligonucleotide plus a further 30b oligonucleotide whose 5′ end corresponded to the desired deletion end point (hence −400, −300 and −100). The mutant p500(KO)CAT was generated by digestion at the *Pst*I site at −213 and resection of the overhangs using T4 DNA polymerase. The resulting blunt-ended plasmid was re-ligated and the desired deletion confirmed by sequence analysis. The plasmid pπ213(3)CAT was derived from pSS0.02CAT by ligation of three copies of the footprint 3 sequence (Figure 3B) into the *Hin*dIII site at −94 within the GST-π promoter. The integrity and orientation of the oligonucleotides (all three in the same orientation as found in the c-*erb*B-2 promoter) were confirmed by sequencing.

Transfection assays

Standard calcium phosphate protocols (Ausubel *et al.*, 1987) were used to introduce *erb*-2−CAT plasmid DNAs (20 μg) into cells at 20−30% confluence. Parallel control transfections contained 20 μg pSV2CAT. All transfections included 5 μg of a β-galactosidase expression plasmid as an internal control. Cells were harvested and standard CAT and Gal assays performed (Ausubel *et al.*, 1987). Scintillation counts for each CAT assay were corrected for transfection efficiency using the Gal reading from the same extract. Corrected counts were compared to parallel transfections using pCATbasic. Each transfection was carried out in duplicate and each set of transfections was repeated at least three times using at least two different plasmid preparations. The mean results are presented plus and minus the standard errors of the mean. Transfection assays were confined to ZR75-1 and T47D cells as only these lines were sufficiently transfectable to allow repeat experiments. The c-*erb*B-2−CAT constructs reached a maximum of 40% of the activity obtained from parallel transfections with SV2CAT in ZR75-1 cells, but this figure was only 5−10% in the low-expressing T47D line. The levels of CAT activity obtained were of the order of 0.07−0.4 nmol [^{14}C]acetyl chloramphenicol/min/mg protein extract for the c-*erb*B-2−CAT constructs in ZR75-1 cells.

Nuclear extracts, footprinting and EMSA assays

Nuclei were prepared from subconfluent cells by lysing them in lysis buffer [0.32 M sucrose, 3 mM $CaCl_2$, 2 mM MgOAc, 0.5% NP40, 10 mM Tris−HCl (pH 7.4), 5 mM dithiothreitol (DTT)]. The nuclei were resuspended in high-salt buffer (0.45 M KCl buffer C; Dignam *et al.*, 1983) to extract nuclear proteins. Extracts were dialysed versus 0.15 M KCl dialysis buffer (Dignam *et al.*, 1983) and the protein concentration estimated (Bio-Rad). Various regions of the c-*erb*B-2 flanking sequence were used as footprinting probes, including the PCR-generated fragments described above, and a further fragment from −497 to −7 also generated by PCR. All of these fragments were cloned into the *Sma*I site of Bluescript (in both orientations) and ^{32}P labelled at the adjacent *Eco*RI site. Probes and an A+G ladder were prepared, and footprinting assays were performed as previously described (Hurst, 1991). For EMSA assays, probes were made using complementary oligonucleotides to the sequences in Figure 3B (with terminal *Hin*dIII sites). These oligonucleotides were annealed, labelled and used in assays as previously described (Hurst, 1991).

Acknowledgements

We are grateful to Ian Goldsmith for the oligonucleotides. We would like to thank our colleagues, Ian Cowell and Malcolm Parker, for reading the manuscript and Zoë Redley for typing it.

References

Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (1987) *Current Protocols in Molecular Biology.* John Wiley & Sons, New York.

Bartek,J., Bartkova,J., Kyprianou,N., Lalani,E.-L., Staskova,Z., Shearer,M., Chang,S. and Taylor-Papadimitriou,J. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 3520−3524.

Cleveland,D.W., Lopata,M.A., MacDonald,R.J., Cowan,N.J., Rutter,W.J. and Kirschner,M.W. (1980) *Cell*, **20**, 95−105.

Cowell,I.G., Skinner,A. and Hurst,H.C. (1992) *Mol. Cell. Biol.*, **12**, 3070−3077.

Dignam,J.D., Lebowitz,R.M. and Roeder,R.G. (1983) *Nucleic Acids Res.*, **11**, 1475−1489.

Gusterson,B.A., Price,K.N., Gelber,R.D., *et al.* (1993) *J. Clin. Oncol.*, in press.

Hudson,L.G., Ertl,A.P. and Gill,G.N. (1990) *J. Biol. Chem.*, **265**, 4389−4393.

Hurst,H.C. (1991) In Collins,M. (ed.), *Methods in Molecular Biology.* Humana Press, NY, Vol. **8**, pp. 141−151.

Hynes,N.E., Gerber,H.A., Saurer,S. and Groner,B. (1989) *J. Cell. Biochem.*, **39**, 167−173.

Inglehart,J.D., Kraus,M.H., Langton,B.C., Huper,G., Kerns,B.J. and Marks,J.R. (1990) *Cancer Res.*, **50**, 6701−6707.

Ishii,S., Imamoto,F., Yamanashi,Y., Toyoshima,K. and Yamamoto,T. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 4374−4378..

Kameda,T., Yasui,W., Yoshida,K., Tsujino,T., Nakayama,H., Ito,M. and Tahara,E. (1990) *Cancer Res.*, **50**, 8002−8009.

King,C.R., Kraus,M.H. and Aaronson,S.A. (1985) *Science*, **229**, 974−976.

King,C.R., Swain,S.M., Porter,L., Steinberg,S.M., Lippman,M.E. and Gelmann,E.P. (1989) *Cancer Res.*, **49**, 4185−4191.

Kraus,M.H., Popescu,N.C., Amsbaugh,C. and King,C.R. (1987) *EMBO J.*, **6**, 605−610.

Kury,F.D., Schneeberger,C., Sliutz,G., Kubista,E., Salzer,H., Medl,M., Leodolter,S., Swoboda,H., Zeillinger,R. and Spona,J. (1990) *Oncogene*, **5**, 1403−1408.

Leask,A., Rosenberg,M., Vassar,R. and Fuchs,E. (1990) *Genes Dev.*, **4**, 1985−1998.

Lemoine,N.R., Barnes,D.M., Hollywood,D.P., Hughes,C.M., Smith,P., Dublin,E., Gullick,W.J. and Hurst,H.C. (1992) *Br. J. Cancer*, **66**, 1116−1121.

Maniatis,R., Fritsch,E.F. and Sambrook,J. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Parkes,H.C., Lillycrop,K., Howell,A. and Craig,R.K. (1990) *Br. J. Cancer*, **61**, 39−45.

Press,M.F., Cordon-Cardo,C. and Slamon,D. (1990) *Oncogene*, **5**, 953−962.

Roberts,S. and Bentley,D. (1992) *EMBO J.*, **11**, 1085−1093.

Slamon,D.J., Clark,G.M., Wong,S.G., Levin,W.J., Ullrich,A. and McGuire,W.L. (1987) *Science*, **235**, 177−182.

Slamon,D.J., Godolphin,W., Jones,L.A., Holt,J.A., Wong,S.G., Keith,D.E., Levin,W.J., Stuart,S.G., Udove,J., Ullrich,A. and Press,M.F. (1989) *Science*, **244**, 707−712.

Suen,T.-C. and Hung,M.-C. (1990) *Mol. Cell. Biol.*, **10**, 6306−6315.

Tal,M., King,C.R., Kraus,M.H., Ullrich,A., Schlessinger,J. and Givol,D. (1987) *Mol. Cell. Biol.*, **7**, 2597−2601.

Ullrich,A., Coussens,L., Hayflick,J.S., *et al.* (1984) *Nature*, **309**, 418−425.

White,M.R. and Hung,M.-C. (1992) *Oncogene*, **7**, 677−683.

Williams,T.M., Weiner,D.B., Greene,M.I. and Maguire,H.C. (1991) *Pathology*, **59**, 46−52.

Wright,C., Nicholson,S., Angus,B., Sainsbury,J.R.C., Farndon,J., Cairns,J., Harris,A.L. and Horne,C.H.W. (1992) *Br. J. Cancer*, **65**, 118−121.

Yamada,Y., Yoshimoto,M., Murayama,Y., Ebuchi,M., Mori,S., Yamamoto,T., Sugano,H. and Toyoshima,K. (1989) *Jpn. J. Cancer Res.*, **80**, 1192−1198.

Yamamoto,T., Ikawa,S., Akiyama,T., *et al.* (1986) *Nature*, **319**, 230−234.

Yan,D.-H. and Hung,M.-C. (1991) *Mol. Cell. Biol.*, **11**, 1875−1882.

Yu,D., Matin,A. and Hung,M.-C. (1992) *J. Biol. Chem.*, **267**, 10203−10206.

Received on December 8, 1992; revised on February 22, 1993