A Heterodimeric Nuclear Protein Complex Binds Two Palindromic Sequences in the Proximal Enhancer of the Human erbB-2 Gene

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Increased expression of the protein-tyrosine kinase receptor ErbB-2 occurs frequently in human breast and ovarian cancer and causes transformation in experimental systems. Control of transcription of the erbB-2 gene is an important determinant of receptor expression. Within the human erbB-2 promoter, a 100-base pair (bp) region 5' to the TATA box enhances transcription 200-fold. Two palindromes present in this 100-bp region are important for both positive and negative transcriptional control. A nuclear palindrome binding protein (PBP) has been purified to near homogeneity using ion-exchange, DNA-affinity, and gel filtration chromatography. PBP is a heterodimer consisting of a 69-kDa α subunit that binds DNA and a 60-kDa β subunit that appears to enhance subunit binding. DNase I footprinting and electrophoretic mobility shift assays indicate that PBP binds to the half-site of each palindrome with the core recognition sequence TGGGAG. By DNA binding specificity and lack of immunological cross-reactivity, PBP is distinct from NF-κB and Ikaros, two proteins with related DNA binding specificities. PBP is proposed to be important regulator of transcription of the erbB-2 gene.

The erbB-2 gene is the second identified member of the EGF receptor subfamily of receptor tyrosine kinases that also includes ErbB-3 and ErbB-4 (1–5). erbB-2 was initially identified as the neu oncogene in rat neuro/glioblastomas induced by transplacental mutagenesis with ethylnitrosourea (6–10). It is oncogene in rat neuro/glioblastomas induced by transplacental mutagenesis with ethylnitrosourea (6–10). It is

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The abbreviations used are: EGFR, epidermal growth factor receptor; neu, an oncogenic mutant of ErbB-2 (Val664 → Glu); PBP, palindrome-binding protein; EMSA, electrophoretic mobility shift assay; bp, base pair(s); PAGZ, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography.

We previously reported the sequence of 3.65 kilobases of the human erbB-2 gene promoter (23, 24). This promoter has typical TATA and CAAT elements in the region proximal to the translation start site (+1) and 4 Alu sequences located in the upstream region (24). A 100-bp region upstream of the TATA box increased basal promoter activity 200-fold (25). This 100-bp region contains a strong Sp1 site near the 5' end and a functional CAAT box near the 3' end. Two palindromic sequences are a prominent feature of this 100-bp control region (23, 26, 27); the distal palindrome (Pal I) overlaps the major Sp1 site and the proximal palindrome (Pal II) overlaps the CAAT box (25). An AP2 site which is located 5' to this 100-bp region increased expression in several breast cancer cell lines that exhibited elevated AP2 activity (21, 28).

Similar palindromes are present in rat, mouse, and human erbB-2 promoters (29). Using reporter constructions, these palindromes were shown to exert both positive and negative regulation of the human erbB-2 promoter (25). To investigate control of the erbB-2 promoter via these palindromic sites, we have purified and characterized a protein complex that specifically interacts with active but not with mutant palindromic sequences. This palindromic binding protein (PBP) is a heterodimer consisting of 69- and 60-kDa subunits. PBP interacts with the half sites of both palindromes I and II primarily via the 69-kDa subunit. Although the palindrome DNA half-sites resemble NF-κB and Ikaros binding sites, nucleotide competition and immunological studies indicate that PBP is distinct from both.

MATERIALS AND METHODS

Oligonucleotides—Oligonucleotides were synthesized using an Applied Biosystems 380 DNA synthesizer and were labeled with [γ-32P]ATP and T4 polynucleotide kinase. The DNA probe corresponding to ~329 to ~230 bp of the erbB-2 promoter was generated by polymerase chain reaction and end-labeled with [γ-32P]ATP and the Klenow fragment of DNA polymerase.

DNA-affinity columns were prepared using established methods (30) in which multimerized double-stranded oligonucleotides were coupled to CNBr-activated Sepharose 4B. WT is the Pal II sequence flanked by TCGA (see Fig. 1). MT is Mb flanked by TCGA. The WT and MT oligonucleotides used for multimerization have the following sequences:

WT: TCGACCTGGAGGCGGCGTTCTCCCCAA
GACCCCTCCGCAAGGCGGTAGGCT

MT: TCGACGGGCGGCGTTGCCCCAA
GACCCCTCCGCAAGGCGGTAGGCT

Cell Culture and Antisera—F9 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The murine pre-B cell line 70Z/3 was cultured in RPMI medium supplemented with 10% fetal bovine serum and 10 μM 2-mercaptoethanol. Anti-p65, anti-Rel, and anti-p50 antisera generated against the respective mouse proteins were generous gifts from Dr. I. M. Verma, the Salk Institute (31). Anti-Ikaros antisera directed against residues 197–431 of Ikaros was a generous gift of Dr. S. Smale, UCLA.

Electrophoretic Mobility Shift Assay and UV-cross-linking—Binding reactions contained 2 × 106 cpm unsubstituted or substituted bromode-
**erbB-2 Promoter Palindrome-binding Protein Complex**

**FIG. 1.** Human erbB-2 promoter. A, diagram of the 100-bp enhancer region of the erbB-2 gene located between −329 and −230 bp relative to the translation start site. The CCAAT box and major Sp1 binding sites are boxed, and the palindromes are indicated with arrows. B, sequences of Pal I, Pal II, and mutant Pal II oligonucleotides that correspond to the translation start site. The CCAAT box and major Sp1, sequences of Pal I, Pal II, and mutant Pal II oligonucleotides that

**FIG. 2.** Purification of the palindrome- binding protein complex. A, aliquots of the indicated fractions from each step of the purification were assayed by EMSA using a Pal II oligonucleotide probe. B, elution of PBP from the Pal II sequence-specific DNA-affinity column as a function of [KCl]. Aliquots of each fraction were assayed using a Pal II oligonucleotide probe. C, Superdex 200 FPLC chromatography of PBP. Fractions were assayed for PBP activity by EMSA using a Pal II oligonucleotide probe. Arrows denote the elution positions of the molecular mass markers (ferritin (440 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and RNase A (14 kDa)). SM, starting material from second WT DNA-affinity column before loading onto the Superdex 200 column. D, protein in the peak of activity from the Superdex 200 column. The predominant 69-kDa and 60-kDa bands are designated as α and β, respectively.
TABLE I

Purification of palindromic binding protein (PBP)

| Step | Fraction | Protein | Total activity | Specific activity | Purification | Recovery |
|------|----------|---------|----------------|------------------|--------------|----------|
| 1    | Nuclear extract | × 10⁻³ μg | × 10⁻⁶ units | units/mg | -fold | % |
| 2    | DE52     | 221     | 78             | 0.35             | 1.2          | 82       |
| 3    | MT DNA affinity | 202     | 64             | 0.32             | 1.1          | 68       |
| 4    | WT DNA affinity 1 | 0.712   | 29             | 40               | 137          | 31       |
| 5    | WT DNA affinity 2 | 0.002   | 10             | 5138             | 17417        | 11       |
| 6    | Superdex 200 | ND      | 7.2            | ND               | ND           | 8        |

Nuclear extracts were negatively absorbed to DE52 and to a DNA-affinity column prepared using an oligonucleotide that retained the central 8 bp but was mutant in both halves of Pal II (Fig. 1). Two sequential site-specific DNA-affinity chromatography steps resulted in approximately a 17,000-fold enrichment of PBP (Fig. 2, A and B, and Table I). When this material was chromatographed on Superdex 200 using FPLC, the peak activity migrated anomalously at a position corresponding to ∼14 kDa (Fig. 2C). As shown in Fig. 2D the peak DNA binding activity consisted of 2 proteins of ∼69 and 60 kDa designated α and β, respectively. A larger M, protein of ∼150,000, which exhibited weak DNA binding activity, was removed by the Superdex 200 chromatography step. A faster migrating band was weakly detected. Purified PBP is thus a tightly associated complex consisting of approximately equimolar amounts of two proteins.

Interaction of PBP with the erbB-2 Promoter—To identify the sites of interaction with the 100-bp erbB-2 core promoter/enhancer region, purified PBP was used for DNase I footprinting (Fig. 3). Four binding sites were identified corresponding to half of each palindrome. The core binding sequence is TGGGAG.

Previous studies indicated that mutations in either the 5′ or 3′ half of the palindromes decreased only modestly the DNA binding and functional activity of the palindromes suggesting that the half-site was the functionally active response element (25). At lower concentrations of PBP, a single complex (Ca) was observed in EMSA on both Pal I and Pal II (Fig. 4). With increasing amounts of PBP, a second slower migrating complex, Cb, was formed. These results support the concept that dimeric PBP binds principally to a half-site core sequence in either palindrome; with higher concentrations of PBP, both half-sites can be occupied simultaneously.

PBP bound to both Pal I and Pal II and DNA binding specificity were confirmed using oligonucleotide competitors in electrophoretic mobility shift assays. As shown in Fig. 5A, binding of PBP to 32P-labeled Pal I was competed by unlabeled Pal I or Pal II oligonucleotides but not by an oligonucleotide containing the 8-bp central region of Pal II, but with alterations in both of the half-sites were identified by DNase I footprinting. Binding was not competed by a consensus Sp1 binding site oligonucleotide or by an NF-κB site oligonucleotide. These results indicate that, although an Sp1 binding site partially overlaps Pal I, PBP does not recognize the Sp1 binding site. While the PBP binding site closely resembles an NF-κB site, PBP did not recognize an NF-κB site derived from the HIV long terminal repeat (36) either by competition (Fig. 5A) or by direct binding assays (data not shown). PBP binding exhibited similar binding specificity for Pal II (Fig. 5B). Binding was competed by mutations in either palindrome half-site (M3, M5, L5M) but not by mutations in both (Mb). These results confirm PBP binding to either half-site of the palindromes.

Binding to DNA via the α Subunit of PBP—Because the dimeric PBP complex bound to a 6-bp core representing half of...
each palindrome, it was important to determine how the complex recognized DNA. PBP was UV-cross-linked to 5-bromo-dUTP-substituted Pal I or Pal II, and the products were analyzed by SDS-PAGE and autoradiography. As shown in Fig. 5, C and D, the 69-kDa α subunit is the major DNA binding unit. A small amount of the 60-kDa β subunit (<10%) was detected bound to Pal I but none was bound to Pal II. DNA binding specificity was confirmed using unlabeled oligonucleotides as competitors in cross-linking assays. Binding of the 69-kDa α subunit was competed by Pal I and Pal II oligonucleotides but not by Mb, NF-κB, or Sp1 oligonucleotides. The small amount of 60-kDa β subunit binding to Pal I was incompletely competed by palindrome oligonucleotides but was competed by the Sp1 oligonucleotide indicating lack of specificity of the β subunit binding to DNA.

The proteins present in the single site EMSA complex formed on Pal I and on Pal II (Fig. 5, A and B) were eluted and separated by SDS-PAGE. Approximately equal amounts of the 69- and 60-kDa species were detected in each complex (Fig. 6A). Thus, although the 69-kDa α subunit was the species cross-linked to the DNA binding site, equal amounts of α and β subunits were present in the DNA-bound complex providing evidence that PBP is a heterodimer.

Southwestern blotting confirmed specific binding of 32P-labeled Pal II to the 69-kDa α subunit of PBP (Fig. 6B). The higher molecular mass band was not competed, consistent with it corresponding to the smaller amount of higher molecular mass activity shown in Fig. 2C, and the 87-kDa band was inconsistent. When Southwestern blotting was carried out using nondenaturing polyacrylamide gel electrophoretic separation of proteins, strong specific DNA binding was observed (Fig. 6B, right panel). This DNA binding activity was at least 100-fold higher using native compared to denaturing gel electrophoresis, a result that could reflect incomplete protein renaturation or higher affinity of the heterodimer αβ PBP compared to the α subunit alone.

Distinguishing PBP from NF-κB and Ikaros—Because the half-sites of the erbB-2 palindromes resemble the DNA recognition elements for NF-κB (Fig. 1) and because NF-κB, like PBP, binds as a heterodimeric protein complex (p65/p50 and rel/p50 (31)), it was important to distinguish PBP from NF-κB. DNA recognition appeared distinct because an NF-κB binding site did not compete with erbB-2 palindromes for PBP binding (Fig. 5) nor did PBP bind directly to the NF-κB site (data not shown). erbB-2 Pal I competed weakly for NF-κB binding to its cognate recognition site (Fig. 7A, lanes 2 versus 4). Antibodies specific for p65, rel, and p50 supershifted the NF-κB complexes formed on the cognate NF-κB binding site oligonucleotide (Fig. 7A, lanes 5, 6, and 7). These antibodies failed to affect PBP complexes formed on either Pal I or Pal II of erbB-2 (Fig. 7B). By both DNA binding specificity and lack of immunological reactivity, PBP thus appears distinct from NF-κB and NF-κB, like PBP, binds to the DNA recognition site of the erbB-2 promoter. A, EMSA analysis of binding of PBP to 5-bromo-dUTP-substituted Pal I without (0) or with a 50-fold excess of the indicated oligonucleotides. comp, competitor. B, EMSA analysis of binding of PBP to 5-bromo-dUTP-substituted Pal II without (0) or with a 50-fold excess of the indicated oligonucleotides. C, UV-cross-linking analysis of binding of PBP to 5-bromo-dUTP-substituted Pal I without (0) or with a 50-fold excess of the indicated oligonucleotides. D, UV-cross-linking analysis of binding of PBP to 5-bromo-dUTP-substituted Pal II without (0) or with a 50-fold excess of the indicated oligonucleotides. Proteins were resolved on 10% SDS-PAGE gels and autoradiographed.

DISCUSSION

Excessive mitogenic signaling via the ErbB-2 receptor tyrosine kinase may result from mutational activation as occurs with rat neu (15) or from gene amplification and enhanced
transcription of wild-type erbB-2 (18–20). Regulation of transcription of the erbB-2 gene is one important determinant of the extent of ErbB-2 expression. It is thus important to identify elements that control transcription of the erbB-2 gene. As assayed using reporter gene constructs in Hela and CV1 cells, full promoter activity of the proximal −1500 bp of human erbB-2 was retained in the −330 bp proximal to the translation start site (23). Several breast cancer cell lines are reported to have strong AP2 activity which increased expression via a response element located at −397 bp (21, 28). A 100-bp strong enhancer region is located proximally at −329 to −230 bp (25). This region contains a 5′ Sp1 site and a 3′ CAAT box. There are 2 dyad symmetries within this 100-bp region that are highly conserved among human, rat, and mouse promoters (29). When placed in front of a minimal TATA box promoter, palindromes enhanced activity. Whereas deletion of Pal I in the context of the erbB-2 promoter reduced activity consistent with an enhancing effect, deletion of the 3′-half of Pal II that overlaps the CAAT box increased promoter activity. Function of the palindromes was thus complex and dependent on their context within the promoter.

A dimeric protein complex that specifically binds these erbB-2 palindromes has been purified more than 17,000-fold. The first indication that PBP exists as a complex was its excessive retention on gel filtration chromatography. The excessive retention of the 69- and 60-kDa proteins on the Superdex 200 column is, perhaps, due to hydrophobic interaction of both proteins with the peak of EMSA activity provided evidence that both participated in the active PBP complex. Approximately equal amounts of the 2 subunits were isolated from the DNA-bound complex separated on EMSA. The 69-kDa α subunit contacted the palindrome half-sites. The amount of the 60-kDa β subunit cross-linked to Pal I was small, less specific, and binding to Pal II was not detected. Only binding to the α subunit was detected by Southwestern analysis. Although we cannot exclude that the β subunit is a proteolytic product of α, this appears unlikely. Approximately equal amounts of α and β were present in the protein complexes bound to both Pal I and Pal II. Moreover, material purified through 2 site-specific DNA-affinity columns and resolved on Superdex 200 contained approximately equal amounts of α and β. Although β did not bind DNA, Southwestern analysis revealed 100-fold higher DNA binding to the native complex than to the α subunit resolved on denaturing gels. While this could be due to incomplete renaturation of the α subunit, the results taken together suggest that PBP is a heterodimeric complex in which binding of the α subunit to DNA is enhanced by the β subunit.

DNase I footprinting confirmed binding to 4 sites within the 100-bp region that correspond to the halves in each palindrome. Each half of the two palindromes appears to be an independent binding site with the sequence TGGGAG. Increasing amounts of PBP resulted in formation of a slower migrating complex on EMSA consistent with occupancy of both halves of the two palindromes.

Cloning will be required to further characterize PBP and its relation to other transcription factors. DNA binding specificity and lack of immunological cross-reactivity indicate PBP is distinct from NF-κB and Ikaros, two proteins with related DNA binding specificity. We suggest that PBP will prove an important regulator of erbB-2 transcription and thus biological responses to this tyrosine kinase growth factor receptor.

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