Regulation of the ERBB-2 Promoter by RBPJk and NOTCH*

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Within the human ERBB-2 gene promoter, a 100-base pair region 5' to the TATA box enhances basal transcription 200-fold. Two palindromes present within this 100-base pair region are important for transcription. The palindrome binding protein was purified to homogeneity and found to be identical to RBPJk, the mammalian homolog of Drosophila Suppressor of Hairless (Su(H)). Recombinant RBPJk bound the ERBB-2 promoter with affinity comparable with that seen with well characterized RBPJk binding sites. RBPJk activated an ERBB-2 palindrome-containing promoter in 293 cells. Because in Drosophila Su(H) acts downstream of NOTCH and because NOTCH-Su(H)/RBPJk stimulates transcription from target promoters, NOTCH-IC, a constitutively active form of NOTCH, was tested for effects on the ERBB-2 palindrome. NOTCH-IC further increased RBPJk-mediated transcription on wild type but not mutant ERBB-2 palindrome. Thus, RBPJk can activate ERBB-2 transcription and serve as an anchor to mediate NOTCH function on the ERBB-2 gene.

We previously described a palindrome binding protein (PBP)† that bound to the half-site of each ERBB-2 palindrome with the core recognition sequence TGGGAG (13). We now report that protein sequence analysis of purified PBP identifies it as RBPJk (recombination signal binding protein of immunoglobulin Jk gene). RBPJk, which was initially cloned by Matsunami et al. (14) based on recognition of the Jk recombination signal sequence, was subsequently isolated based on recognition of the Epstein-Barr virus (EBV) C promoter (designated as CBF1) (15–17) and of the adenovirus pIX gene promoter (18). RBPJk is the mammalian homolog of Drosophila Suppressor of Hairless (Su(H)) (19, 20). Further analysis indicated that although RBPJk contained a 40-amino acid region of homology to integrase, it lacked such activity (21) and recognized a composite sequence consisting of the heptamer recombination recognition site and a BamHI linker (15, 22). RBPJk is widely expressed with two mouse and three human splicing variants identified (23).

Genetic analyses indicate that Su(H) acts downstream of NOTCH in the signaling pathway of sensory peripheral nervous system development in Drosophila (19, 20). A similar pathway for lateral inhibition in neuronal development exists in mammalian species (24). The Drosophila Enhancer of split (E(spl)) and the mouse hairy enhancer of split (Hes-1) complexes are downstream of NOTCH and Su(H)/RBPJk in this pathway (25, 26). In the best studied mechanism, the intracellular domain (IC) of the transmembrane protein NOTCH is translocated to the nucleus with RBPJk that serves as the site-specific DNA binding partner (26, 27). The NOTCH-IC-RBPJk complex activates transcription of basic helix loop proteins encoded by Hes-1. Events downstream of E(spl)/Hes-1 may be inhibitory (lateral inhibition in neuronal development (28) and inhibition of myogenesis (29)) or stimulatory. RBPJk also acts in a protein complex with the non-DNA-binding EBNA2 to regulate genes involved in EBV latency (15). The alteration of NOTCH that is necessary for nuclear translocation is reported to occur upon ligand binding (27) via proteolysis (30), via translational delay of Hes-1 (31), and with retroviral insertion (32).

Because RBPJk could bind to the two palindromes in the ERBB-2 promoter, we examined its activity using reporter gene constructs. We present evidence that RBPJk stimulates promoter activity via wild type but not mutant ERBB-2 palindromic sequences; activity was markedly enhanced upon co-

* The abbreviations used are: PBP, palindrome binding protein; bp, base pair(s); EBV, Epstein-Barr virus; Su(H), Drosophila Suppressor of Hairless; Hes-1, Hairless enhancer of split; E(spl), Drosophila Enhancer of split; IC, intracellular domain; HA, hemagglutinin; Pal I, distal palindrome; Pal II, proximal palindrome; Mb, Pal I mutated in both sites; EMSA, electrophoretic mobility shift assay; TES, 2-[2-hydroxyethyl]ethylenediaminetetraacetic acid; HPLC, high pressure liquid chromatography; wtPal II-TK, wild type Pal II thymidine kinase; mutPal II-TK, mutated Pal II thymidine kinase; Hes-TK, Hes-1 thymidine kinase.

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expression of the intracellular domain of NOTCH but was not affected by co-expression of EBNA2. These results suggest that ERBB-2 promoter activity is regulated by a protein complex that contains RBPJκ.

MATERIALS AND METHODS

Cell Cultures, Transfections, and Reporter Assays—293 cells were grown in Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 10% calf serum. F9 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Plasmid DNA was transfected as a calcium phosphate precipitate (33). A β-galactosidase expression vector under control of a cytomegalovirus promoter was co-transfected with the luciferase reporter gene constructs.

Luciferase activity was measured as described by de Wet et al. (34). Aliquots of cell extract were added to an assay reaction containing 100 mM potassium phosphate (pH 7.8), 5 mM ATP, and 15 mM MgSO4 in a volume of 0.35 ml. Reactions were initiated by the addition of 0.1 ml of 1 mM luciferin, and light readings were integrated over 10 s with a monolight 2110 luminometer. β-Galactosidase activity was measured as described by Norton and Coffin (35). Promoter activity was expressed as light units of luciferase activity/mg of protein.

Hayward, The Johns Hopkins University. The NOTCH-IC and RBPJκ cDNAs were generated by polymerase chain reaction and subcloned into a modified pCDNA3 vector containing the luciferase gene translational initiation codon and the hemagglutinin (HA) epitope tag. The intracellular region of human NOTCH (NOTCH-IC) was a generous gift from Dr. David Baltimore, MIT. Viral EBNA2 and human RBPJκ cDNAs were generous gifts from Dr. Diane Hayward, Johns Hopkins University. The NOTCH-IC and RBPJκ cDNAs were regenetrated by polymerase chain reaction and subcloned into a modified pCDNA3 vector containing the luciferase gene translation initiation codon and the hemagglutinin (HA) epitope tag.

Fig. 1. Nuclear protein binding to the palindromes of the ERBB-2 promoter. EMSA was carried out with 32P-labeled 100-bp ERBB-2 oligonucleotide probe (329–230 relative to the translation start site at +1) incubated with F9 nuclear extract without (0) or with a 100-fold excess of unlabeled Sp1 or Pal II oligonucleotides. Positions of the Sp1 and PBP complexes are indicated by arrowheads. comp, competitor.

The mass spectrum contained one strong signal at m/z = 1280. The other fraction was Q(TP)(AV)(QL)(DP)(AV)(DT)DL(IV)VSQIL (Fraction 46) where residues given in parentheses were observed in the same sequencing cycle, indicating the presence of two peptides in that fraction. The mass spectrum contained a major signal at m/z = 1235 and a weak signal at m/z = 1750. A BLAST computer homology search (38) revealed that all these sequences were identical to trypic fragments of mouse/Xenopus RBPJκ. All molecular masses determined were within 0.05% of the masses calculated for the corresponding RBPJκ trypic fragments.

RESULTS

Palindromes of the ERBB-2 Gene Interact with a Specific Nuclear Protein, PBP—Previous studies showed that a 100-bp region upstream of the TATA box of the ERBB-2 gene promoter increased basal promoter activity 200-fold (11). Two palindromic sequences are a prominent feature of this 100-bp enhancer element in addition to a strong Sp1 site near the 5′ end and a CAAT box near the 3′ end. The distal palindrome (Pal I) overlaps the Sp1 site, and the proximal palindrome, Pal II, overlaps the CAAT box (8, 9). When this 100-bp region was used as a probe in EMSA, proteins in F9 nuclear extract formed several complexes (Fig. 1). The complexes that could be effectively competed by an Sp1 consensus sequence are represented by Sp1 and proteins with a recognition motif similar to Sp1. A complex designated as PBP could not be competed by the Sp1 consensus oligonucleotide but was effectively competed by the palindromic sequence. F9 cells thus contain a specific palindromic binding activity.

PBP Is RBPJκ—To determine the identity of PBP, it was purified to homogeneity from F9 cells using ion exchange, DNA affinity, and gel filtration chromatographies and SDS-polyacrylamide gel electrophoresis and then transferred onto a nitrocellulose membrane. After visualization by staining with Amido Black 10B, the protein band was excised and subjected to sequencing analysis. The mass spectrum contained one strong signal at m/z = 1280. The other fraction was Q(TP)(AV)(QL)(DP)(AV)(DT)DL(IV)VSQIL (Fraction 46) where residues given in parentheses were observed in the same sequencing cycle, indicating the presence of two peptides in that fraction. The mass spectrum contained a major signal at m/z = 1235 and a weak signal at m/z = 1750. A BLAST computer homology search (38) revealed that all these sequences were identical to trypic fragments of mouse/Xenopus RBPJκ. All molecular masses determined were within 0.05% of the masses calculated for the corresponding RBPJκ trypic fragments.

To verify that RBPJκ had palindromic binding activity, the protein was expressed as a HA-tagged fusion protein and transfected into 293 cells. Transient expression of HA-RBPJκ resulted in a large increase of PBP activity that could be competed by wild type Pal II but not by Mb oligonucleotides. An
indicating that flanking sequences significantly affect RBPJk ERBB-2 gene promoter (8). It is, however, a poor competitor, perfect matching consensus site is located at 2
bound to the J
drome. A 22-bp 32P-labeled Pal II probe was incubated with nuclear extract made from 293 cells or from 293 cells transiently transfected with HA-RBPJk. Reactions were incubated without (0) or with a 100-fold excess of the indicated unlabeled competitors or with a monoclonal antibody against the HA tag. Ab, antibody; comp, competitor; WT, wild type Pal II; MT, mutant Pal II.

antibody against the HA tag supershifted the complex, supporting the sequence identification of PBP as RBPJk (Fig. 3).

Binding of PBP Is Compe ted E ffectively by Ot her RBPJk Binding Sites—RBPJk was originally isolated as a protein that bound to the Jk recombination signal sequence consisting of a heptamer and a nonamer with a 23-bp spacer (14). RBPJk protein is highly conserved from Caenorhabditis elegans to Homo sapiens (14, 15, 19, 20, 39). The consensus recognition sequence for the RBPJk family has subsequently been determined as GTGGGAA, which is present in all RBPJk binding sites characterized so far (22). The PBP core site CTGGGAGC is close but not identical to the consensus sequence (13). To compare the affinity of ERBB-2 palindromes with other RBPJk binding sites, palindromes as well as other characterized binding sites were used as unlabeled competitors in EMSA using partially purified F9 nuclear extract and Pal II as a probe. As shown in Fig. 4, the EBV Cpo sequence is the optimal binding site. Pal I, Pal II, and half-site mutation M3 have affinity similar to that seen with the Hes-1 and pIX gene promoters. A perfect matching consensus site is located at -596 of the ERBB-2 gene promoter (8). It is, however, a poor competitor, indicating that flanking sequences significantly affect RBPJk binding affinity for the core consensus sequence.

RBPJk-stimulated Tran script ion via the ERBB-2 Palin drome Is Augmented by NOTCH-IC but Not by EBNA2—To study the function of RBPJk on the ERBB-2 promoter palindrome, RBPJk was transfected into 293 cells along with reporter constructs containing wild type or mutant palindrome sequences (Fig. 5). The control reporter TK was induced 3-fold by RBPJk. RBPJk activated wtPal II-TK 16-fold, whereas mut-Pal II-TK was induced no more than the background activation seen with the vector TK. Constitutively active forms of mammalian NOTCH stimulated transcription of the Hes-1 gene presumably through RBPJk and its binding site on the Hes-1 promoter (26). The intracellular domain of NOTCH was transfected to determine whether an active form of human NOTCH (NOTCH-IC) had any effect on the ERBB-2 gene (Fig. 5). NOTCH-IC alone did not increase reporter activity above background. However, co-transfection of NOTCH-IC with RBPJk activated wtPal II-TK activity 82-fold. NOTCH-IC failed to affect the basal stimulation of RBPJk seen with TK and mutPal II-TK. The effect of NOTCH-IC was thus specific to the wild type ERBB-2 palindrome-containing gene and was dependent on RBPJk.

The effects of RBPJk and NOTCH-IC on ERBB-2 palindromes were compared with effects on the Hes-1 promoter. As shown in Fig. 6, NOTCH-IC alone was sufficient to induce activation of the Hes-1 promoter, whereas RBPJk alone did not stimulate the Hes-1 reporter. Co-transfection of RBPJk and NOTCH-IC caused no change in activity beyond that observed with NOTCH-IC alone. EBNA2 is a well described activator of some RBPJk response genes (15, 36). It up-regulates Cpo and CD23 genes utilizing RBPJk and its binding sites on extended promoter elements. When EBNA2 was transfected into 293 cells, it had no effect on ERBB-2 or Hes-1 promoter activity. EBNA2 also had no effect on activation mediated by RBPJk and/or NOTCH-IC on either the ERBB-2 palindromes or the Hes-1 site. Thus EBNA2 requires interactions additional to those mediated via RBPJk (40), whereas effects of
NOTCH-RBPJk occur on promoters containing only RBPJk response elements.

**DISCUSSION**

The present studies identify the protein (PBP) that binds to the two palindromes of the proximal enhancer of ERBB-2 as RBPJk. Peptide sequences of tryptic fragments of PBP were identical to those of RBPJk (14–16). Although the ERBB-2 palindrome half-sites do not perfectly match the reported RBPJk consensus binding sequence (22), PBP/RBPJk bound to the palindromic sites with an affinity similar to that observed for the consensus sites. NFκB/Rel binding sites, which also resemble the palindrome half-sites, do not compete for PBP binding (13). Additionally, nuclear extracts containing epitope-tagged RBPJk formed a specific complex with ERBB-2 palindromes. Previous studies identified two components of PBP (13). We suggest that these likely represent splicing variants of RBPJk with varying N termini (23).

RBPJk is reported to function as a site-specific DNA binding protein that recruits additional transcription factors to target genes (17, 36, 41). RBPJk represses transcription from its cognate site in the adenovirus pIX gene promoter (18) and from a Gal4 binding site when expressed as a Gal4 fusion (42). A repression domain in RBPJk was identified that coincided with that required for activation by EBNA2 (42). EBNA2 was thus deduced to activate transcription by both masking the repression domain of RBPJk and by bringing a strong transactivation domain to the RBPJk binding site.

RBPJk is the mammalian homolog of Drosophila Su(H), which functions in the NOTCH pathway and regulates development of the peripheral nervous system (19, 20). Su(H) interacts positively with NOTCH (43, 44) and negatively with Hairless (45). Fortini and Artavanis-Tsakonas (27) observed that Su(H) was translocated to the nucleus when the transmembrane receptor NOTCH was activated by binding the ligand Delta. Interestingly, an oncogene from a human T lymphoblastic leukemia (TAN-1) was identified as a truncated NOTCH lacking much of the extracellular domain (31). Kopan et al. (30) presented evidence that proteolytic processing of NOTCH could generate NOTCH-IC sufficient to act via a complex with RBPJk in the nucleus. RBPJk binds to the intracellular domain of NOTCH, and expression of RBPJk with ectodomain-deleted forms of NOTCH (δEC-NOTCH), both with and without retention of the transmembrane domain, activates transcription from Hes-1 and EBV C promoters (26, 46). δEC-NOTCH-RBPJk and EBNA2/RBPJk complexes exhibited similar transcriptional enhancement on EBV C promoter sites (46).

RBPJk stimulated transcription from a reporter gene containing wild type but not mutant ERBB-2 palindrome sequences. This activity was greatly enhanced by NOTCH-IC. Using immunofluorescence, we found that NOTCH-IC and RBPJk localize in the nuclei of 293 cells (data not shown). These observations support models of RBPJk/NOTCH-IC heterodimers that act to enhance transcription from RBPJk response elements. Comparison of ERBB-2 palindrome and Hes-1 elements in reporter gene assays indicates a stronger effect of transfected RBPJk alone on ERBB-2 and a stronger effect of transfected NOTCH-IC alone on Hes-1. This may reflect a higher affinity in vivo of endogenous RBPJk for the Hes-1 site. RBPJk was not inhibitory on either site. On these sites, EBNA2 had no effect. This suggests that stimulatory effects of EBNA2

![Fig. 5. Stimulation of ERBB-2 promoter palindrome II by RBPJk and NOTCH-IC. 293 cells were transfected with 2μg of the indicated reporter constructs, 3μg of RBPJk, 1.5μg of NOTCH-IC, and 0.1μg of cytomegalovirus-β-galactosidase expression plasmids. Relative activity is the ratio of normalized luciferase activity in the presence of the indicated expression vectors divided by the activity in the presence of the parental vector pCDNA3 and in the absence of any expression plasmid. Normalized luciferase activity equals light units of luciferase activity/Δ_{280} unit of β-galactosidase activity. The numbers are the mean ± S.D. (n = 3), and the experiment was repeated 3 times with similar results. When not shown, error bars are within the column lines.](image)

![Fig. 6. Lack of effect of EBNA2 on Hes-1 and ERBB-2 palindromes. 293 cells were transfected with 1.5μg of the indicated reporter constructs, 2μg of RBPJk, 1.5μg of NOTCH-IC, 1.3μg of EBNA2, and 0.2μg of cytomegalovirus-β-galactosidase expression plasmids. Data are presented as in Fig. 5, and the experiment was repeated 2 times with similar results.](image)
require interaction with additional proteins such as Spi-1/Spi-B, which bind to a more extended EBV C element (40).

Several recognition elements have been identified within the extended ERBB-2 promoter. Two AP2 and two Sp1 consensus binding sequences are found at −397, −359, −369, and −314, respectively, relative to the translation start site (8). Several breast cancer cell lines are reported to have a strong AP2 activity that increases expression via the response element located at −397 bp (47). A stimulatory factor that binds to the promoter region 3′ of the TATA box has also been described (48). Analyses of the rat ERBB-2 promoter have identified a transacting factor that binds at −466 to −456 bp (49) and inhibition by co-expression of Rb (50), adenovirus E1A (51), and c-Myc (52). However, deletional analysis of the human ERBB-2 gene identified the region between −329 and −230 bp as the major strong enhancer region; it contains two functionally important palindromes (11). The activity of RBPJx, which binds to each palindrome half-site (13), is strongly augmented by NOTCH signaling pathways that are known to be important in neuronal development (14).

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