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Biochemistry

A mutant neomycin phosphotransferase II gene reduces the resistance of transformants to antibiotic selection pressure

(selectable marker/chimeric gene)

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ABSTRACT **The neo (neomycin-resistance) gene of transposon Tn5 encodes the enzyme neomycin phosphotransferase II (EC 2.7.1.95), which confers resistance to various aminoglycoside antibiotics, including kanamycin and G418. The gene is widely used as a selectable marker in the transformation of organisms as diverse as bacteria, yeast, plants, and animals. We found a mutation that involves a glutamic to aspartic acid conversion at residue 182 in the protein encoded by the chimeric neomycin phosphotransferase II genes of several commonly used transformation vectors. The mutation substantially reduces phosphotransferase activity but does not appear to affect the stability of the neomycin phosphotransferase II mRNA or protein. Plants and bacteria transformed with the mutant gene are less resistant to antibiotics than those transformed with the normal gene. A simple restriction endonuclease digestion distinguishes between the mutant and the normal gene.**

The aminoglycoside phosphotransferases belong to a clinically important group of proteins that enable bacteria to grow in the presence of selected aminoglycoside antibiotics, including kanamycin, neomycin, and G418. These enzymes, which are plasmid or transposon encoded, inactivate the antibiotics by catalyzing the transfer of the terminal phosphate of ATP to the drug (1). Neomycin phosphotransferase II (NPTII; EC 2.7.1.95) is one of the most well-known members of this group. The neomycin-resistance (neo) gene of Tn5 that encodes this enzyme is the most widely used selectable marker in the transformation of organisms including yeast (2), *Dictyostelium discoideum* (3), mammals (4), and plants (5). The gene is usually modified to contain a promoter and terminator that can function in the recipient host. Cells that receive and properly express such chimeric constructs can be selected by using antibiotic concentrations that inhibit the growth of cells not containing the resistance gene.

The aminoglycoside 3′-phosphotransferases exhibit 30–36% amino acid sequence homology except in a region near the carboxyl terminus, where the homology is 60–70% (6). It has been suggested that the catalytic functions of binding ATP and transferring the terminal phosphate are performed at the more conserved region (7). We have found a mutation in this region of chimeric NPTII genes of several widely used transformation vectors. The mutation causes reduced NPTII enzyme activity, resulting in a decreased ability of transformants to grow in the presence of elevated concentrations of antibiotic.

MATERIALS AND METHODS

Chemicals and Reagents. Antibiotics and *p*-nitrophenyl phosphate were purchased from Sigma. Purified NPTII protein, rabbit antibody to NPTII, and biotinylated rabbit antibody to NPTII were generously supplied by Edward Halk (Agrigenetics). Streptavidin-alkaline phosphatase conjugate was from Bethesda Research Laboratories. The plant transformation vector pBI121 (8) is sold by Clontech. The restriction endonuclease *Xho* II and the mammalian transformation vector pMC1neo (9) were purchased from Stratagene. [α-^{32}P]dATP and [γ-^{32}P]ATP were purchased from New England Nuclear. Corning disposable ELISA plates and P 81 phosphocellulose paper were from Whatman. Sequenase was from United States Biochemical. Nitrocellulose was purchased from Schleicher & Schuell. Sequence-specific oligonucleotide primers were purchased from The DNA Factory (San Diego, CA).

General Methods. Plasmid DNA isolation, restriction digestion, ligation, nick-translation of probes, and Southern and Northern analyses were carried out as described by Maniatis *et al.* (10). Polysomal RNA from tobacco was isolated according to Goldberg *et al.* (11). ELISA for NPTII was performed according to Firoozabady *et al.* (12). The procedure for tobacco leaf disc transformation follows that of Horsch *et al.* (13).

NPTII Activity Assay. The phosphorylation of kanamycin was assayed by the agarose gel overlay procedure of Reiss *et al.* (14), after the electrophoretic separation of proteins in clarified tobacco extracts that had been prepared by homogenization in 50 mM Tris·HCl, pH 7.4/10% (vol/vol) glycerol/2.5% (vol/vol) 2-mercaptoethanol/0.1% SDS.

Construction of Bacterial Plasmids Containing the Normal and the Mutant NPTII Gene. pDAE, a plasmid containing the normal NPTII gene, was constructed by inserting a 1.5-kilobase (kb) *Hin*dIII–*Sal* I fragment obtained from Tn5 into the corresponding sites of PiAN7. The 1.5-kb fragment contains the NPTII coding region and the bacterial promoter and terminator regions (15). To construct a plasmid containing a mutated NPTII gene, a 332-base-pair (bp) *Fsp* I–*Nco* I fragment, nucleotides 1779–2111 (16), containing the mutation at nucleotide position 2096 was removed from the chimeric mutant NPTII gene of the plant transformation vector pCIB10 (17) and inserted into pDAE cut with the same enzymes. The resulting plasmid was designated pDAD. The structure of the mutant gene was verified by DNA sequencing. *Escherichia coli* strain HB101 was transformed with pDAE and pDAD. Transformants were selected on kanamycin (20 μg/ml) and identified by restriction digestion of minilysate DNA.

RESULTS

Detection of a Mutation in the NPTII Gene. In experiments involving the transformation of tobacco tissues, we routinely used vectors containing various chimeric NPTII gene con-

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Abbreviations: NPTII, neomycin phosphotransferase II; neo gene, neomycin-resistance gene.

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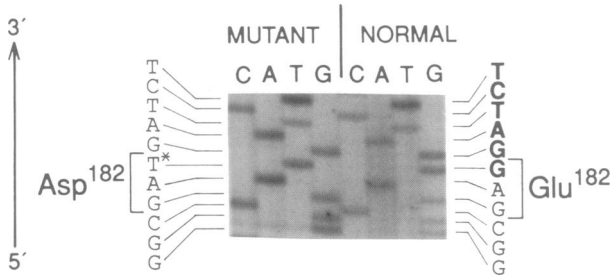

FIG. 1. Partial nucleotide sequence comparison between the normal and mutant NPTII genes. The genes were subcloned into PiAN7 and sequenced by the dideoxynucleotide chain-termination method, using T7 DNA polymerase. Asterisk indicates site of point mutation. Boldface type designates *Xho* II recognition site. The first guanosine in the *Xho* II site corresponds to nucleotide position 2096 (16).

structs. In the course of measuring the amount and activity of NPTII, we found that plants transformed with some of the vectors displayed a negative correlation between the amount and activity of the enzyme. To investigate the possibility that the reduced enzyme activity may result from a defect in the gene, we sequenced a chimeric NPTII gene encoding an enzyme with low phosphotransferase activity (Fig. 1). Compared to the sequence of the normal gene (16), a single change at nucleotide position 2096 was found, where the guanosine is replaced with a thymidine residue. This converts codon 182 from GAG (glutamic acid) to GAT (aspartic acid).

Function of the Normal and Mutant Enzyme in Plants. When leaf discs from tobacco plants were cultured on various concentrations of G418, discs containing the normal gene showed 50% inhibition of growth at a G418 concentration of 30–40 μg/ml, compared with 50% inhibition at a G418 concentration of 9 μg/ml for leaf discs containing the mutant gene (Fig. 2). Control leaf discs lacking a selectable marker showed 50% inhibition of growth at a G418 concentration of 3 μg/ml. The decreased resistance of leaf discs containing the

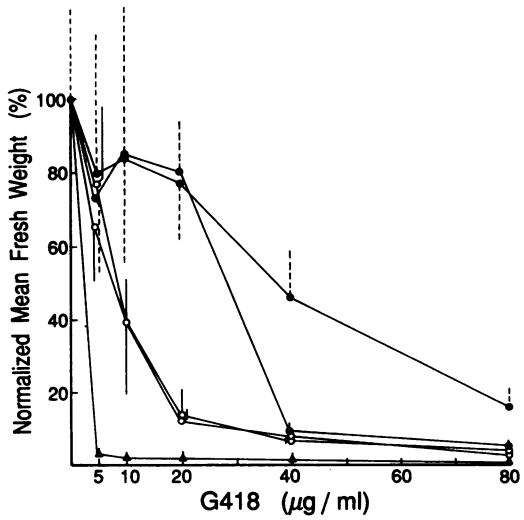

FIG. 2. Tobacco leaf disc assay for antibiotic tolerance. Leaf discs from tobacco plants containing a mutant or normal NPTII gene were cut out and cultured on MS medium supplemented with benzyladenine at 0.75 mg/liter and the indicated amounts of G418. The remaining leaf material was used for ELISA (see Table 1). Twenty discs per transformant were used for each concentration of G418. Leaf disc fresh weight was determined after 28 days. The results are expressed as the percent of leaf disc weight relative to the weight of discs from the same plant plated in the absence of antibiotic. Triangles, nontransformed tobacco control; open circles, transformants containing a mutant gene; solid circles, transformants with a normal gene.

Table 1. NPTII protein amounts in transgenic tobacco leaf tissue

Chimeric NPTII gene	NPTII, ng/mg of total soluble protein
None (control)	0
Normal (plant 1)	3
Normal (plant 2)	10
Mutant (plant 1)	160
Mutant (plant 2)	440

Samples were from the same leaves that were used for the antibiotic tolerance assay in Fig. 2. NPTII was determined by ELISA. Total protein was determined by the Bradford protein assay (Bio-Rad).

mutant gene is not due to a decrease in the level of gene expression. NPTII protein amounts were determined in the leaf tissues used for preparing the leaf discs shown in Fig. 2. The results, shown in Table 1, demonstrated that plants containing the mutant gene had higher levels of NPTII protein than plants containing the normal gene (160–440 ng of NPTII per mg of total soluble protein versus 3–10 ng of NPTII per mg, respectively).

Fig. 3 shows the results of assays of several parameters in tobacco plants transformed with a normal or mutant chimeric NPTII gene. Leaf extracts from plants containing the normal enzyme (Fig. 3*A*, lanes 1–3) showed a higher NPTII phosphorylating activity than that of extracts containing the mutant enzyme (Fig. 3*A*, lanes 4 and 5). On the other hand, Northern blot analysis of polysomal RNAs from the same tissues (Fig. 3*B*) revealed low NPTII mRNA levels in plants with high NPTII enzyme activity and high levels of mRNA in plants with undetectable or barely detectable enzyme activity (compare lanes 1–3 with lanes 4 and 5 in Fig. 3 *A* and *B*). ELISA analysis (Fig. 3*C*) showed that NPTII protein amounts correlated positively with mRNA levels but negatively with enzyme activity.

Function of the Normal and Mutant Enzyme in Bacteria. The experiments discussed above were carried out with chimeric NPTII constructs modified to function in plant systems. To

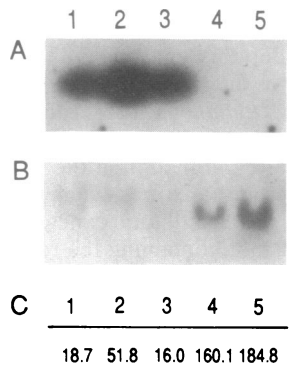

FIG. 3. NPTII enzyme activity, mRNA, and protein in transgenic tobacco plants. Leaf tissue was ground in liquid nitrogen and portions of the frozen powdered material were assayed for NPTII enzyme activity (*A*), NPTII mRNA (*B*), and NPTII protein (*C*). (*A*) *In situ* NPTII enzyme activity assay. Plant extracts were subject to nondenaturing polyacrylamide gel electrophoresis, and the gel then overlayed with 1% agarose containing [γ-^{32}P]ATP and kanamycin. Enzyme activity was assayed by autoradiography after the transfer of phosphorylated kanamycin to phosphocellulose paper. Lanes: 1–3, extracts from plants containing the normal protein; 4 and 5, extracts containing the mutant protein. (*B*) Northern blot analysis. Total polysomal RNA (20 μg) from the same transformants as in *A* was probed with a 1.1-kb nick-translated NPTII gene fragment. (*C*) Quantitation of NPTII protein in tobacco leaf extracts. Total protein was determined using the Bradford protein assay (Bio-Rad). NPTII protein was quantitated using an ELISA. Results are expressed as ng of NPTII per mg of total soluble protein. Samples 1–5 are as in *A* and *B*.

provide further support for our finding that the mutation has a negative effect on the activity of the NPTII enzyme, we proceeded to introduce the mutation into a bacterial vector. The wild-type Tn*5* neo gene was inserted into PiAN7 and altered by replacing a segment of the gene with the corresponding segment derived from a mutant chimeric gene. This introduced a single-base-pair change in the DNA sequence of the neo gene at nucleotide position 2096. After transformation of *E. coli*, we compared the function of the altered and normal enzymes in bacteria (Fig. 4). Both enzymes conferred resistance to kanamycin at 100 and 200 μg/ml. However, at 400 μg/ml, bacteria containing the mutant enzyme displayed retarded growth compared to bacteria carrying the normal enzyme (Fig. 4 *A* and *B*). The difference in growth rate was more dramatic when G418 was used as the selective agent. Cells containing the normal enzyme detoxified G418 at all concentrations tested, while cells containing the mutant enzyme exhibited poor growth in G418 at 20 μg/ml and no growth in G418 at 30 μg/ml during a 9-hr culture period (Fig. 4 *C* and *D*). With the bacterial cultures, as seen with the plant cultures, the decreased resistance is not due to decreased expression of the mutant gene. ELISA performed on the starting cultures indicated the presence of 18.5 and 27.6 ng of NPTII protein per μg of total soluble protein for the normal and mutant enzyme, respectively.

Occurrence of the Mutation in Diverse Chimeric NPTII Gene Constructs. As noted earlier, we observed that a number of plant transformation vectors appeared to have an NPTII-selectable marker displaying low phosphotransferase activity. We decided to analyze the NPTII gene constructs present in a variety of plant and animal transformation vectors to see if the mutant gene was widely spread. We made use of the fact that the mutation resides within an *Xho* II recognition site. In a normal NPTII gene there are two *Xho* II sites in the coding region of the gene, at nucleotide positions 1850 and 2096. An additional *Xho* II site is present in the 3′ noncoding region at position 2488 (16). Digestion of the gene with *Xho* II produces a diagnostic 246-bp fragment from within the coding region. If the NPTII 3′ noncoding region containing the *Xho* II site is present in the structure of the chimeric gene, an additional 392-bp fragment is generated. As indicated in Fig. 1, the G→T substitution at nucleotide 2096 results in the loss of an *Xho* II site. Therefore, digestion of the mutant chimeric gene with *Xho* II will produce a 638-bp fragment in place of the 246- and 392-bp fragments. In Fig. 5 are the results of a Southern blot analysis of a variety of transformation vectors digested with *Xho* II and probed with a labeled NPTII fragment. The bands labeled A, B, and C represent the 246-, 392-, and 638-bp fragments, respectively. The first six lanes contain digests of plant transformation vectors. Fragments A and B are present

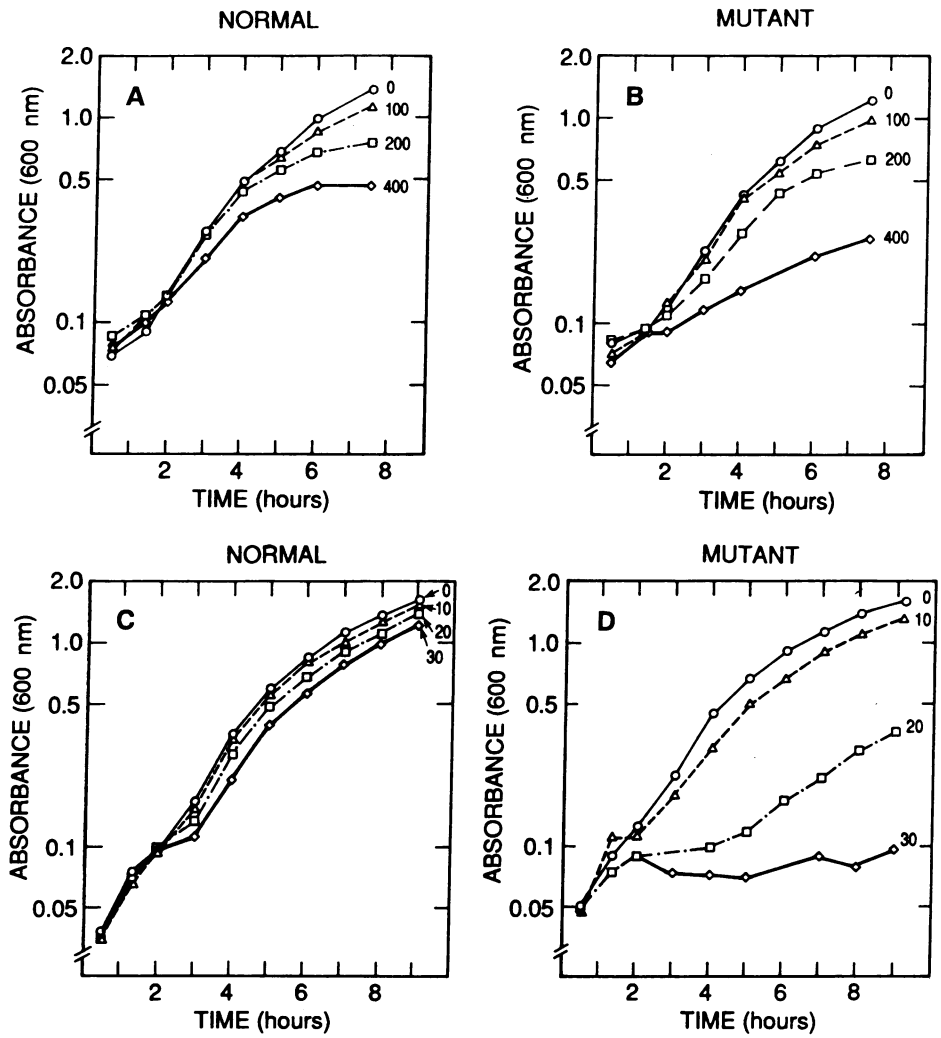

FIG. 4. Effect of antibiotics on the growth of *E. coli* transformed with plasmids containing the normal or mutant neo gene. Bacterial transformants containing plasmids pDAE and pDAD were grown to stationary phase in the presence of kanamycin at 20 μg/ml. A portion of each culture was removed for NPTII ELISA analysis (see text) and the remainder was diluted 1:100 into medium containing the indicated amounts of kanamycin (*A* and *B*) or G418 (*C* and *D*). The cultures were grown with constant shaking at 37°C. Numbers to the right of the growth curves reflect the amount of antibiotic present, in μg/ml. The absorbance at 600 nm was measured at the indicated times.

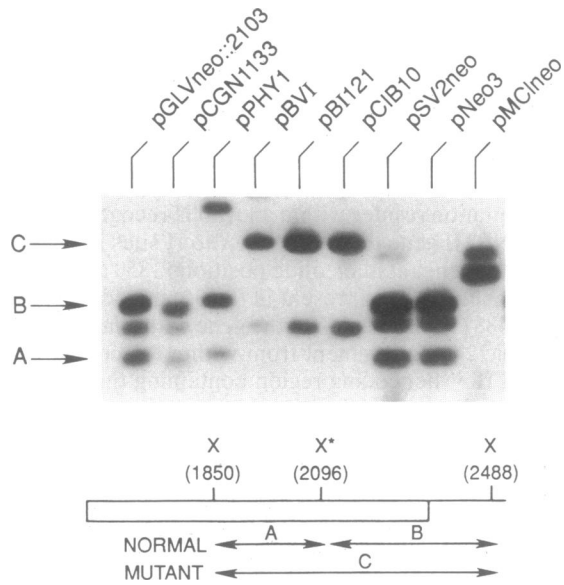

FIG. 5. Southern blot analysis of vectors containing NPTII constructs. All plasmids were digested with *Xho* II and probed with a 1.1-kb *Pst* I–*Eco* RI NPTII–NOS fragment from pCIB10 (17). (*Upper*) Fragments A, B, and C are 246, 392, and 638 bp, respectively. (*Lower*) Schematic diagram of *Xho* II sites present in the DNA sequence of the NPTII gene. Open box, NPTII coding region; thin line, 3′ noncoding region. X, *Xho* II restriction site. Numbers in parentheses refer to nucleotide position in the DNA sequence (16). Asterisk indicates location of point mutation.

in pGLVneo::2103 (18), pCGN1133 (unpublished data, Calgene, Davis, CA), and pPHY1 (unpublished data, Phytogen, Pasadena, CA), indicating that they contain the normal gene. Mutant genes, as indicated by the absence of fragments A and B and the presence of fragment C, are found in pBVI (19), pBI121 (8), and pCIB10 (17). The next three lanes contain digests of vectors used in mammalian systems. pSV2neo (20) and pNeo3 (21) contain the normal gene. pMC1neo (9) contains a mutant gene as indicated by the absence of fragment A. Fragment C is not present in the pMC1neo digest because the chimeric construct does not contain the NPTII 3′ noncoding region. We have sequenced the region containing nucleotide 2096 in pMC1neo. The mutation is the same G → T substitution found in the plant transformation vectors (results not shown).

DISCUSSION

Our results demonstrate the presence of a mutation in the NPTII gene serving as the selectable marker in a number of widely used transformation vectors. The mutation is brought about by a G → T substitution at nucleotide position 2096 in the neo gene of Tn*5* and results in the encoding of aspartic acid in place of the glutamic acid at position 182 of the amino acid sequence. The presence of the mutant enzyme results in a decreased ability of tobacco leaf discs to grow on elevated concentrations of the antibiotic G418, as compared with the normal enzyme. At the same time, the transformants containing the mutant enzyme had substantially higher levels of the mutant protein, suggesting that the mutant gene encodes a protein with significantly reduced phosphotransferase activity. The comparison of NPTII amounts and enzyme activity levels in the normal and mutant protein in tobacco leaf tissues further substantiates that the phosphotransferase activity is significantly reduced in the mutant. The results observed in the plant tissues were further corroborated by showing that the growth of *E. coli* containing the mutant gene was also retarded in the presence of elevated levels of antibiotics. The higher NPTII mRNA and protein levels seen in tobacco leaf tissue containing the mutant as opposed to the normal gene probably reflect differences in transcriptional activity and not altered stability of the mutant mRNA or protein product. This assumption is based on the fact that in some tobacco transformants containing the mutant NPTII gene, we have observed the positive correlation between the NPTII mRNA and protein when they are present at low levels (results not shown).

Observations of homologies between the amino acid sequences of kinases and phosphotransferases have led to the suggestion that both classes of proteins have catalytic domains of similar function and general location within the structure of the protein (7). Biochemical and mutational analyses have shown that the catalytic domain of the protein kinases resides in the carboxyl-terminal portion of the protein (for reviews, see refs. 22 and 23). The conserved region of amino acid sequence shared by the phosphotransferases and the kinases localizes the catalytic domain of the phosphotransferases to the carboxyl terminus. The invariant aspartic acid and asparagine residues, present in all phosphotransferases (7) and kinases (24), are found here. The glutamic acid residue at position 182 in NPTII is found in this region and is conserved in all known type II aminoglycoside 3′-phosphotransferases (25).

No mutations have been described that affect the biochemical behavior of the NPTII or related phosphotransferase enzymes. Using chemical modification of amino acid residues in NPTII followed by analysis of enzyme activity, J. P. Greer and M. H. Perlin (personal communication) have implicated histidine-188 with the binding of kanamycin and tyrosine-218, -244, or -257 with the binding of ATP.

On a practical level, the reduced activity of the mutant NPTII enzyme clearly affects the ability of transformants to withstand antibiotic selection pressure from kanamycin or its analogs. A properly functioning selectable marker is critical for maximizing the potential for detection of the few transformed cells in a population after a transformation procedure. Based on our analysis of plant and animal transformation vectors, it is likely that many laboratories, working in diverse disciplines, are using a mutant NPTII gene encoding a phosphotransferase of low activity. Digestion with the endonuclease *Xho* II provides a simple test to quickly determine if the mutation is present in the NPTII gene.

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