The EMBO Journal vol.3 no.13 pp.3317-3322, 1984

Protein fusions with the kanamycin resistance gene from transposon Tn5

Bernd Reiss[1], Rolf Sprengel and Heinz Schaller

Department of Microbiology, University of Heidelberg, Im Neuenheimer Feld 230, 6900 Heildelberg, FRG

[1]Present address: Department of Biochemistry, Biological Sciences West, University of Arizona, Tucson, AZ 85721, USA

Communicated by H.Schaller

The gene for the neomycin phosphotransferase II (NPT II) from transposon Tn5 was fused at the amino or carboxy terminus to foreign DNA sequences coding for 3-300 amino acids and the properties of the fused proteins were investigated. All amino-terminal fusions examined conferred kanamycin resistance to their host cell, but profound differences in their enzymatic activity and stability were detected. Short additions to the amino terminus of the NPT II resulted in highly enzymatically active fusion proteins whereas long amino-terminal fusions often had to be proteolytically degraded to release active proteins. Fusions at the carboxy-terminal end of the NPT II protein did not always induce kanamycin resistance and their enzymatic activity depended more stringently on the nature of the junction sequence.

Key words: expression in *E. coli*/fusion proteins/neomycin phosphotransferase II/protein processing

Introduction

The *neo* gene from transposon Tn5 encodes a neomycin phosphotransferase II (NPT II) and therefore confers resistance to a host cell towards aminoglycoside antibiotics like kanamycin (Km), gentamycin and neomycin. The gene has been extensively used as an expression and selection marker in many different hosts; in prokaryotes like *Escherichia coli, Bacillus subtilis* and *Myxococcus* (Rao and Rogers, 1979; Sprengel, 1983; Shimkets *et al.*, 1983), in lower eukaryotes including *Dictyostelium* (Hirth *et al.*, 1982), in animal cells (Colbére-Garapin *et al.*, 1981; Southern and Berg, 1982) and in plant cells (Herrera-Estrella *et al.*, 1983; Bevan *et al.*, 1983).

The *neo* gene and its control signals have been well characterised (Beck *et al.*, 1982) and suitable and sensitive enzymatic assays are available to quantitate the NPT II and to characterise modifications of the enzyme (Reiss *et al.*, 1984). Besides its use as a selectable marker, the gene is thus also very attractive for use as a generally applicable indicator gene to examine gene expression and gene regulation. For such studies it would be of advantage if the NPT II could be fused onto a gene product of interest without losing enzymatic activity. If so, it would be possible to study the regulation of gene expression of such genes and the functioning of their regulatory signals by monitoring the NPT II activity of appropriate protein fusions. In addition, the fused gene product itself could be selected for, independent of the host systems in use.

To provide an experimental basis for this approach, we have examined a variety of N-terminal NPT II fusion proteins in *E. coli* cells with respect to their expression, their ability to confer kanamycin resistance (KmR) to the host and their enzymatic activity. In addition, several C-terminal NPT II fusions were constructed and analysed for their potential usefulness as selectable marker proteins.

Results and Discussion

Amino-terminal fusions

We described previously the primary structure of the *neo* gene from Tn5 and also of two N-terminal deletion mutants pKm21 and pKm22, in which one or four of its 264 codons are replaced by synthetic *Hind*III linkers, respectively (Beck *et al.* 1983). These mutants allow fusions into the coding region of the *neo* gene in two reading frames and, after further modification, also into all three possible reading frames relative to a *Bam*HI recognition sequence (Figure 1). These constructs were used to generate NPT II fusions with altered N termini by combining them with different prokaryotic expression units consisting of a promoter, a ribosome binding site and a beginning of the structural gene.

As outlined in Figure 2, three different expression elements were used: (i) the control region of the *lac* operon including the first seven codons of the *lacZ* gene; (ii) the signals from the CAT gene from transposon Tn9 including 73 N-terminal codons of this gene; and (iii) segments of different size from the *penP* gene from *B. licheniformis* which is also highly active in *E. coli* (Sprengel, 1983). A fourth synthetic expression unit was generated by combination of a *lac* promoter element without the beginning of the *lacZ* gene and an oligonucleotide linker providing an initiation codon at the appropriate distance to a cryptic Shine-Dalgarno sequence (SD-sequence; Shine and Dalgarno, 1974) located on the *lac* promoter element. The structure of the chimeric NPT II proteins was further varied by the insertion of DNA fragments from FMDV cDNA (Kurz *et al.*, 1981) between the *lac* expression unit and the residual *neo* gene.

In this way, 10 different gene fusions were obtained which were expected to express NPT II-like proteins with 4-312 amino acids fused to the N terminus of the NPT II (Figures 2 and 3). The pBR322 derivatives carrying these constructs were transferred into *E. coli* and single isolates were analysed for the synthesis of chimeric NPT II proteins and their en-

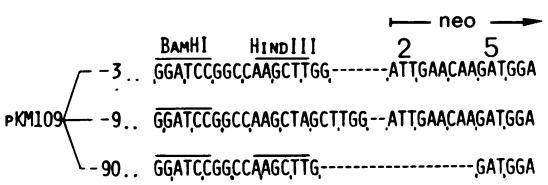

Fig. 1. Nucleotide sequence of *lac/neo* gene junctions in plasmids encoding 5'-truncated *neo* genes appropriate for N-terminal fusions in all three reading frames. The *neo* gene reading frame, the positions of the 2nd and 5th amino acid codon in the authentic *neo* gene and important restriction sites are indicated.

zymatic activity using the following techniques: the plasmid-encoded protein synthesis was quantitated on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) either directly from crude cell extracts by Coomassie blue staining, or after radioactive labeling of plasmid-encoded proteins in the maxicell system (Sancar *et al.*, 1979). NPT II activity induced in *E. coli* cells was roughly determined by growing the bacteria on plates containing Km in concentrations varying from 10 μg/ml to 1000 μg/ml. In addition, in most cases, NPT II levels in the cells were more accurately determined using an improved version of the enzymatic *in vitro* assay for NPT II activity using toluenized cells (Materials and methods). These results were substantiated by assaying for the phosphorylation of Km by proteins separated by PAGE under non-denaturing conditions (NPT II-ndPAGE assay; Reiss *et al.*, 1984). This assay was also used to compare side by side the NPT II activity of the different fusion proteins and of their processing products. Results obtained are presented in Figure 4B and summarised in context with the various constructs in Figure 2.

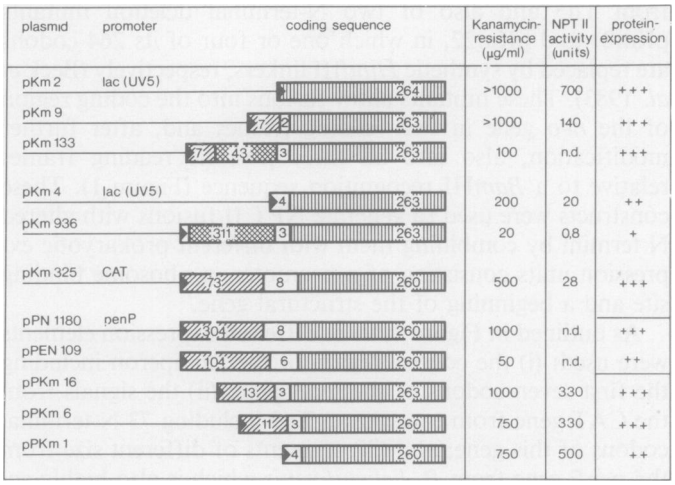

Fig. 2. Schematic diagram of N-terminal fusion proteins illustrating their protein composition. Striped amino acid sequences derived from the *neo* gene, hatched from *lacZ* or *penP* expression units, double hatched from FMDV sequences and white from linker sequences. The length of distinct protein parts in chimeric *neo* genes and names of the corresponding plasmids are included as well as an estimation of the protein synthesis and the induced enzymatic activity of NPT II fusion proteins in *E. coli* cells.

In most cases the amount of NPT II-like protein synthesised reflected directly the strength of the expression system. Strong protein bands of the expected mol. wt. were observed upon SDS-PAGE in crude cell extracts with constructs using the *penP* expression signals (Figure 4A, panel 3), and also in maxicell experiments if the *neo* gene was fused to the authentic *lacUV5* and CAT expression unit. Much less NPT II was obtained with plasmid pKm4 and pKm936 (Figure 4A, panel 1) whose relatively inefficient expression is due to a poor SD-sequence formed by the fusion of *lac* sequences and synthetic linkers (Figure 3).

Short amino-terminal NPT II fusion proteins

As expected, all plasmids listed in Figure 2 conferred KmR to their host cells, but the level of this resistance varied greatly. In general, the short fusions induced high levels of resistance whereas longer fusions showed only moderate to low resistance to the antibiotic, even in cases where substantial amounts of the fused protein were radiolabeled, as determined in the maxicell system (Figure 4B). This already suggests that the specific activity of the NPT II fusions decreases with increasing number of amino acids fused to the N terminus of the enzyme.

Some minor variations in the enzymatic activity were also observed in some of the highly active short NPT II fusions. This is exemplified by a comparison of the results obtained with plasmids pKm2 and pKm9 which express the authentic *neo* gene or a short *lacZ/neo* fusion under the control of the *lac* UV5 promoter (Figure 2, panel 1). As shown in the maxicell experiment (Figure 4B), similar amounts of protein were synthesised in both cases and also a similar level of KmR (>1 mg/ml) was determined in the plating assay (Figure 2). However, upon closer inspection in the *in vitro* NPT II assay, the enzymatic activity induced by the *lac* fusion, and therefore also its specific activity, was found to be ~5-fold lower (Figure 2). This change must be related to the N-terminally added nine amino acids, since the *lac*/NPT II fusion protein was present in the cell extracts in its predicted slightly increased size and, as such, enzymatically active as demonstrated by the comparison with the authentic NPT II in SDS-PAGE and

Fig. 3. Junction sequences of gene fusions encoding the N-terminal NPT II fusion proteins shown in Figure 2. The names of the plasmids and the N-terminal amino acid sequence of the NPT II fusion is given. The N terminus of the authentic NPT II protein is represented by pKm2. The 5′ deletion endpoints of the *neo* genes in pKm21 and pKm22 are indicated and the putative SD-sequences underlined.

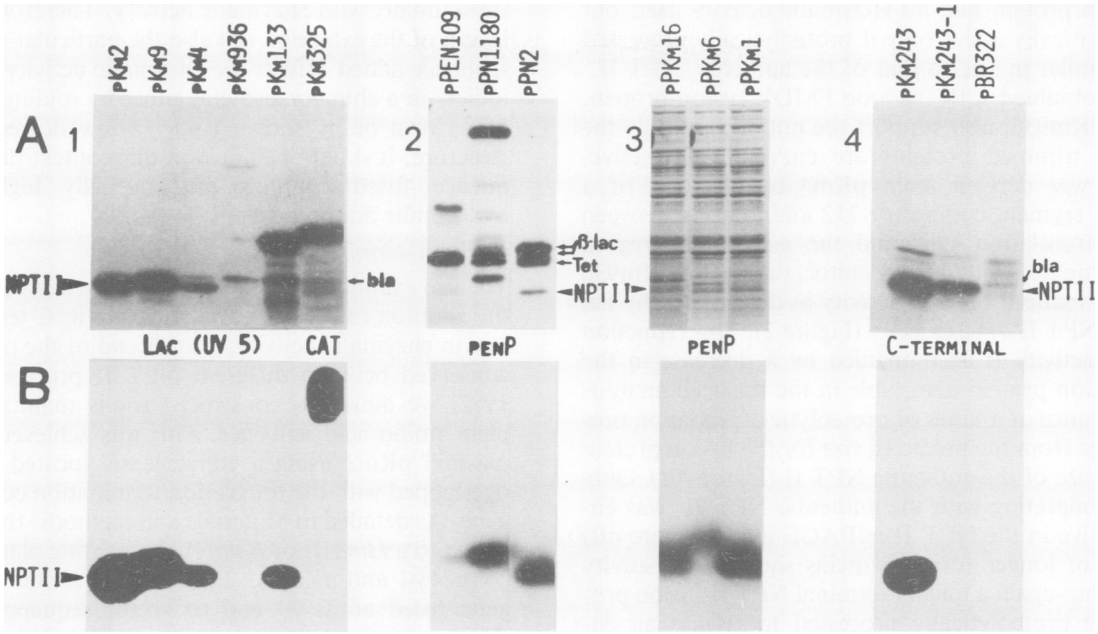

Fig. 4. Analysis of N- and C-terminal NPT II fusion proteins by (**A**) SDS-PAGE and (**B**) NPT II-ndPAGE assay. (**A**) **Panel 1, 2, 4.** Plasmid-encoded proteins were labelled with [^{35}S]methionione in maxicells and analysed in SDS-PAGE (10%). Positions of authentic NPT II, of *penP*-encoded authentic β-lactamase precursor (β-lac), of pBR322-encoded penicillinase precursor (bla) and of pJKK3-1-encoded Tet protein (Tet) are indicated. The authentic Tet protein, NPT II and β-lactamase encoded by pPN2 (Sprengel, 1983) were used as references. **Panel 3.** Crude cell extracts from short β-lactamase/NPT II fusions separated by SDS-PAGE (10%) after Coomassie blue staining. The position of the authentic NPT II is indicated. (**B**) Crude cell extracts used in **A** were analysed by NPT II-ndPAGE assay (see Materials and methods). The position of the authentic NPT II is indicated. In lane pKm936 a longer exposure showed an additional band in the position of the authentic NPT II.

the NPT II-ndPAGE assay (Figure 4A, B).

A detailed analysis of the short *penP/neo* fusions pPKm1, pPKm6 and pPKm16, also indicates differences in the specific activities of the chimeric enzymes: SDS-PAGE analysis of crude cell extracts (Figure 4A, panel 3) revealed that the two constructs containing additional 11 (pPKm6) and 13 (pPKm16) N-terminal codons from the *penP* gene express higher levels of NPT II-like proteins than fusion pPKm1 which adds only one amino acid to the NPT II and whose gene product was not visible in the SDS-PAGE analysis. The increased expression obtained from pPKm6 and pPKm16 is most likely due to the maintenance of the fully intact bacterial ribosome binding site in these constructs which include nucleotides following the AUG initiation codon (Stormo *et al.*, 1982). Regarding the NPT II activity expressed (Figure 2), these increased protein yields are most likely compensated by a loss in specific activity caused by the addition of extra N-terminal amino acids. For pPKm6 this interpretation is supported by the NPT II-ndPAGE assay showing this NPT II fusion as a distinct band of different electrophoretic mobility and of lower enzymatic activity than the pPKm1 fusion (Figure 4B). However, in the same assay a radioactive smear indicates that, for the closely related pPKm16 protein, only degradation products and not the fusion protein itself are enzymatically active. This different behavior of the pPKm13 and pPKm16 fusions may be related to the fact that these were derived from *neo* gene variants which differ at their N-terminal junction sequence by three amino acids which may influence the stability and activity of the fusion protein.

Long amino-terminal fusion proteins

In contrast to the short fusions, the longer fusions showed no simple correlation between protein expression and KmR. This is mainly due to the fact that most of the longer fusion proteins are not enzymatically active as such and had to be converted into products with NPT II activity by proteolytic cleavage.

	DdeI	resistance
pKm2	CTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCA LeuLeuAspGluPhePhe***	> 1000
pKm243	CTTCTTGACGAGTTCTTC CAAGCTTTAATGCGGTAGTTTATCACAGTTAAA LeuLeuAspGluPhePhe GlnAlaLeuMetArg***	> 1000
pKm243/T	CTTCTTGACGAGTTCTTC CAAGCTTTAAATGTTAATTTGCCCTTGGAC LeuLeuAspGluPhePhe GlnAlaLeuAsnValAsnLeuProLeuAsp +253 aa	0
pKm243/t	CTTCTTGACGAGTTCTTC CAAGCTTAGCAGCTGAAAAACAGTTTACAGATG LeuLeuAspGluPhePhe GlnAla***	> 1000
pKm243/Tet	CTTCTTGACGAGTTCTTC CAAGCTTGGCCGGATCCTCTACGCCGGACG LeuLeuAspGluPhePhe GlnAlaTrpProAspProLeuArgArgThr +293 aa	200
pKm243-2/Tet	CTTCTTGACGAGTTCTTC CCGGATCCTCTACGCCGGACGCATCGTGGC LeuLeuAspGluPhePhe ProAspProLeuArgArgThrHisArgGly +290 aa	20
pKm243-1	CTTCTTGACGAGTTCTTC CCAAGCTTTAATGCGGTAGTTTATCACAGTTAA LeuLeuAspGluPhePhe ProSerPheAsnAlaValValTyrHisSer***	0

Fig. 5. Junction sequences of gene fusions encoding C-terminal NPT II fusion proteins. Names of plasmids, length and initial sequence of C-terminal fused amino acids are given. The C terminus of the authentic NPT II is represented by pKm2. KmR levels of the *E. coli* host cells are included.

An example providing evidence for this interpretation is the NPT II fusion pKm133. This construct contains a 43-codon spacer from foot-and-mouth disease virus (FMDV) cDNA between the authentic *lac* translation start and the *neo* gene of pKm9. In the maxicell experiment the corresponding fusion protein was as efficiently expressed as that of pKm9 and synthesized in its full size (Figure 4A). Nevertheless, the potential to direct KmR was very low (~100 μg/ml) compared with the short *lac* fusion pKm9 which was synthesized in similar amounts but conferred KmR of the order of 1 mg/ml.

This difference between the two constructs is explained by the results of the NPT II-ndPAGE assay (Figure 4B) which showed that the *lac* fusion of pKm9 represents an enzymatically active fusion protein, whereas there was no enzymatic activity detected at positions expected for the pKm133 fusion. The only enzymatically active protein found was indistinguishable from the authentic NPT II. Thus the

pKm133 fusion protein had no enzymatic activity itself but became enzymatically active only if proteolytically processed to a protein similar in size to that of the authentic NPT II.

The results obtained with the long FMDV fusion protein, expressed by pKm936, also support the notion that only the proteolytically trimmed proteins are enzymatically active. This plasmid was derived from pKm4 by insertion of a FMDV cDNA segment coding for 312 amino acids between the synthetic translation start and the *neo* coding region (Figure 2). Compared with its progenitor, the fusion pKm936 induces only a reduced NPT II activity as determined by the KmR and the NPT II *in vitro* assay (Figure 2). This reduction of enzymatic activity is accompanied by a decrease in the amount of fusion protein detectable in the maxicell analysis and the appearance of a series of proteolytic degradation products that range from the predicted size for the fusion protein (64 kd) to the size of the authentic NPT II (Figure 4B). Only one band, co-migrating with the authentic NPT II, was enzymatically active in the NPT II-ndPAGE assay (Figure 4B) whereas a set of longer fusion proteins showed no activity (Figure 4B). Thus again a long N-terminal NPT II fusion protein had to be proteolytically processed to release an enzymatically active protein indistinguishable from the authentic NPT II.

Further evidence supporting the necessity for a rather precise proteolytic trimming of long N-terminal NPT II fusions was obtained with two long fusions to the β-lactamase which contain nearly the complete *penP* coding sequence (304 out of 307 amino acids; pPN1180) or the first third of the β-lactamase (pPEN109) (Figure 2). In this case only the longer fusion induced a high level of KmR and NPT II activity. This unexpected result is explained by the rapid degradation of the long fusion pPN1180 giving rise to two prominent proteins similar in size to the authentic β-lactamase and the NPT II, whereas no degradation products of the shorter fusion became visible in the maxicell experiment (Figure 4A). However, the levels of short fusion protein (pPEN109) detected were lower than those of the long fusion (pPN1180), indicating that the short fusion is probably also in part proteolytically degraded to a series of products not visible as distinct bands. Nevertheless, in both cases, only degradation products with identical electrophoretic mobility showed a major NPT II activity in the NPT II-ndPAGE assay (Figure 4B) supporting the hypothesis that the different degree of precision in proteolytic trimming causes the difference in the resistance level observed. In addition, the NPT II-ndPAGE assay of the longer fusion revealed a minor enzymatically active protein of much lower electrophoretic mobility, indicating that longer NPT II fusion proteins may also be enzymatically active.

The CAT fusion protein examined represents a noteworthy exception within the set of longer NPT II fusion proteins. This fusion adds 81 amino acids to the N terminus of the NPT II and confers resistance to high levels of Km (500 μg/ml). The protein is efficiently expressed in its expected size (Figure 4A) and is itself enzymatically active, as the NPT II-ndPAGE assay shows an electrophoretic behavior of an enzymatically active protein as expected for the fusion (Figure 4B). Upon prolonged exposure of the autoradiogram of this assay a series of minor enzymatically active degradation products could be detected ranging in size down to the *lac* fusion of pKm9 (not shown) which indicates that also partial degradation products of the CAT/NPT II fusion were enzymatically active. These results show that exceptional cases exist where an extended N terminus does not interfere substantially with enzymatic activity. Therefore, not only the length of the extension but also the particular structure of the sequence added affects the enzymatic activity of the NPT II moiety in a chimeric protein, either by folding into a domain of its own or by showing only a low degree of secondary structure. It should be noted in this context that the CAT sequence fused contains an unusually high fraction of hydrophilic amino acids.

Carboxy-terminal fusion proteins

We also examined whether the NPT II enzyme would tolerate the addition of foreign amino acids to its C terminus without loss in enzymatic activity. As this end of the protein is highly conserved between different NPT II proteins (Beck *et al.*, 1982) we aimed to construct fusions maintaining its complete amino acid sequence. This was achieved starting from plasmid pKm2 using a conveniently located *Dde*I site that overlapped with the translation termination codon of the *neo* gene. As detailed in Materials and methods, this site was converted to a *Hind*III or *Bam*HI site creating plasmids pKm243, pKm243-1 and pKm243-2/Tet, which contain the intact *neo* gene fused at its 3′ end to vector sequences as expected (Figure 5). Starting from these, other plasmids containing C-terminally modified NPT II proteins (also listed in Figure 5) were obtained by addition of synthetic adapter sequences and of differently truncated coding sequences derived from the SV40 early region (Tooze, 1981) or from the Tet gene of plasmid pBR322 (Suttcliffe, 1978) thus creating a set of short and long fusions with additional 2, 5, 10, 263, 300 and 303 amino acids, respectively. The resulting constructs were compared with respect to KmR in *E. coli* (Figure 5) and some of them for the size and activity of the NPT II hybrids synthesized (Figure 4A and B).

Although not as detailed as compiled for the N-terminal fusions in Figure 2, these constructs show that both long and short C-terminal fusions may lead to active or inactive NPT II proteins. Short fusions adding two amino acids (pKm243/t) or five amino acids (pKm243) were highly active, another one adding 10 amino acids (pKm243-1) was not (Figure 4B), even though it was expressed in maxicells stably and equally as well as the pKm243 fusion (Figure 4A). This suggests that the change in the amino acid sequence following the NPT II from Gln-Ala-Leu to Pro-Ser-Phe may have drastically influenced the activity of the enzyme. This interpretation is supported by a comparison of the results from the two long fusions pKm243/Tet and pKm243-2/Tet which link the NPT II to 299 amino acids of the Tet protein encoded in pBR322. The two constructs differ only in the linking sequence: pKm243-2/Tet is fused *via* a Pro residue whereas pKm243/Tet is fused *via* Gln-Ala-Trp-Pro. This difference of three inserted amino acids results in a drastic increase in enzymatic activity from 20 μg/ml in pKm243-2/Tet to 200 μg/ml in pKm243/Tet, again suggesting that a neighbouring Pro residue may directly or indirectly disturb the enzymatically active structure of the NPT II.

Another long fusion plasmid, pKm243/T, encodes the C-terminal 262 amino acids of the SV40 T-antigen fused to the full length NPT II enzyme encoded in pKm243. This plasmid did not induce any KmR in *E. coli* suggesting that long C-terminal fusions may be inactive even if linked by the same Gln-Ala-Leu sequence that does not interfere with the NPT II activity in a short fusion like pKm243, and which is also very similar to the Gln-Ala-Trp linkage in the active NPT II/Tet fusion encoded in pKm243/Tet.

Concluding remarks

Gene fusions leading to enzymatically active chimeric proteins were first described for the *E. coli* β-galactosidase by Müller-Hill and Kania (1974), and *in vivo* and *in vitro* techniques have been employed to construct and to select for many β-galactosidase fusions. The results described in this report indicate that our terminally modified NPT II may be used in an analogous manner, but without being limited to *E. coli* as a host. In addition, we describe changes in the enzymatic activity and the stability of the NPT II-like proteins induced by the added amino acid sequences, whereas there have been only fragmentary reports on such changes for the β-galactosidase (Zabeau and Stanley, 1982; Rüther and Müller-Hill, 1983). For the NPT II the enzymatic activity of a fused protein seems to be influenced by two factors, namely the size and the amino acid sequence added. In N-terminal fusions the negative influence of leading amino acid sequences was found to increase mainly with their length, unless a folding of the fusion protein allowed a processing and release of enzymatically active degradation products. In contrast, the enzymatic activity of the C-terminal fusions seem to depend much more on the amino acid sequence at the junction. This difference is in keeping with the finding that the *neo* gene tolerates extensive deletions at its 5′ end (J. Davies, personal communication) but not at its 3′ end (Beck *et al.*, 1982). The preliminary data presented suggest that especially a proline residue in the immediate vicinity of the C terminus of the NPT II seems to lead to inactive proteins irrespective of the length of the fusion.

The fact that not all fusion proteins were enzymatically active and that active NPT II can easily be monitored quantitatively and qualitatively opens an experimental approach to select for and to examine amino acid sequences which do not interfere with the proper folding of the NPT II and/or which facilitate correct processing to the active enzyme. The use of such optimized junction sequences should allow us, in principle, to obtain essentially identical, enzymatically active degradation products for any fusion protein constructed. It should also allow the insertion and fusion of the coding region of the *neo* gene in-phase at both ends into reading units of interest with the hope of always obtaining NPT II active products from the chimeric gene. If so, the *neo* gene could be used as universal probe to analyse the activated state of genes. Studies with our present *neo* constructs in several plant cell systems (J. Schell, H.J. Bohnert, B. Hohn and M. Van Montagu, personal communications) and in monkey cells (Reiss, unpublished) recently demonstrated the universal applicability of enzymatically active NPT II fusions.

Materials and methods

Plasmid constructions

All molecular cloning procedures were performed according to Maniatis *et al.* (1982).

Plasmids encoding neo genes with a deleted start codon

In plasmids pKm22 and pKm21 the first or the first four amino acid codons of the *neo* gene are deleted and replaced by a synthetic *Hind*III linker (Beck *et al.*, 1982). Further restriction sites providing the opportunity to fuse the *neo* gene to all possible reading frames were introduced in front of the *neo* gene as follows: starting from pKm22, pKm109/3 was created by substitution of the *Eco*RI/*Hind*III *lac* promoter fragment with a shorter *lac* promoter fragment (Weiher and Schaller, 1982) which contains an additional *Bam*HI site immediately in front of the *Hind*III site. The phasing of the *Bam*HI restriction site relative to the *neo* reading frame was altered by 'filling in' the sticky *Hind*III ends with DNA polymerase I (Backman *et al.*, 1976) and re-ligation to create pKm109/9. To obtain the *Bam*HI site in the third frame the *Hind*III/*Sal*I *neo* gene fragment of pKm109/3 was substituted for the *Hind*III/*Sal*I *neo* gene fragment of pKm21 creating pKm109/90 (see Figure 1).

Plasmids encoding N-terminal NPT II fusion proteins

LacZ fusions. pKm9 was derived from plasmid pKm22 by filling in the sticky *Hind*III ends with DNA polymerase I and re-ligation. For the construction of pKm4 a *Bcl*I linker fragment (CATGATCATG; a gift from H. Seelinger, Ulm) was inserted by blunt end ligation into *Hind*III-linearized pKm109/3 after treatment with nuclease S1. In pKm4, a 936-bp *Sau*3A fragment from the FMDV cDNA clone pFMDV1034 (positions 3045–3981; Kurz *et al.*, 1981) was introduced into this *Bcl*I site thus creating pKm936. This fragment encodes a continuous reading frame for most of the viral coat protein VP1 plus the beginning of a non-structural protein (p14). pKm133 was obtained from pKm936 by exchanging the *lac* promoter and the first two thirds of the FMDV fragment up to an internal *Hind*III site (position 3848) against the *Eco*RI/*Hind*III *lac* promoter fragment of pKm22.

CAT fusions. pKm325 was obtained from pBR325 (Bolivar, 1978; Prentki *et al.*, 1981) by exchange of the 1857-bp *Eco*RI/*Sal*I fragment containing the C-terminal part of the CAT gene against a 1150-bp *Eco*RI/*Sal*I fragment containing the *neo* gene from pKm21 plus the following oligonucleotide linker sequence 5′ added: (5′ GGAATTCCCCGGATCCGGCCAAGCTAGCTTG 3′; Reiss, 1982).

penP fusions. pPKm1, pPKm6 and pPKm16 were obtained as follows. The *penP* gene of *B. licheniformis* as cloned in phage fd/pen1500-2 (Neugebauer *et al.*, 1981) was digested to a limited extent with exonuclease *Bal*31 (Beck *et al.*, 1982) at the *Pst*I site located 16 amino acids downstream from the initiation codon of the *penP* gene. After addition of *Hind*III linkers and restriction nuclease cleavage, *Eco*RI/*Hind*III fragments of ~250 bp containing the promoter and translational start site of the *penP* gene were isolated and ligated simultaneously in-phase to the *neo* gene provided by a mixture of *Hind*III/*Sal*I *neo* gene fragments derived from pKm21 and pKm22 and the *Eco*RI/*Sal*I cleaved *B. subtilis/E. coli* shuttle vector pJKK3-1 (Kreft *et al.*, 1983). The ligation mixture was transferred into *E. coli* and plasmids directing high levels of KmR were analysed by sequence analysis. Three of those isolates are represented by pKm1, pKm6 and pPKm16. Plasmid pPEN109 was constructed using again the *Eco*RI/*Sal*I linearized vector pJKK3-1. Now, however, the authentic *Eco*RI/*Bgl*II *penP* fragment (Neugebauer *et al.*, 1981) encoding the *penP* expression signals and the N-terminal 104 amino acids of the β-lactamase was inserted together with the *Bam*HI/*Sal*I *neo* gene fragment of pKm109/9. pPN1180 was obtained in an analogous way except that an *Eco*RI/*Bam*HI *penP* gene fragment was used which contains, in addition to the expression signals, 304 out of the 307 amino acid codons of the *penP* gene (Sprengel, 1983).

Plasmids encoding C-terminal NPT II fusions

Plasmids pKm243, pKm243-1 and pKm243-2/Tet were derived from pKm2 (Beck *et al.*, 1982). After digestion with *Dde*I the DNA fragments were treated by nuclease S1 to render the 5′-overlapping ends flush and added to a mixture of *Hind*III and *Bam*HI linkers. The ligated DNA was cleaved with *Eco*RI/*Bgl*II *or Eco*RI/*Hind*III and fragments containing the *neo* gene were then recloned into the expression vector pEX150 (Weiher, 1982) *via* the appropriate restriction sites. Plasmid pKm243/T was constructed by exchange of the *Hind*III/*Bam*HI fragment of pKm243 for a similar fragment of SV40 DNA (943 bp; position 3476–2533; Tooze, 1981) containing part of the T-antigen coding region. pKm243/t was obtained by insertion of another *Hind*III/*Bam*HI fragment of SV40 DNA (825 bp; position 1708–2533) into pKm243. Plasmid pKm243/Tet was obtained by exchange of the *Hind*III/*Sal*I fragment of pKm243 for an analogous fragment of pBR322/AD16 containing the *Hind*III/*Bam*HI adapter AD16 (Schaller, unpublished) inserted into the *Bam*HI site of pBR322.

Sequencing procedure

DNA sequencing was performed essentially as described by Maxam and Gilbert (1980). Occasionally sequencing gels were dried in order to enhance band sharpness and to shorten exposure times (Garoff and Ansorge, 1981).

Labeling of plasmid-encoded proteins in maxicells

U.v.-irradiated and starved *E. coli* CSR603 cells (Sancar *et al.*. 1979) transformed with the plasmids indicated were incubated for 40 min with [^{35}S]-methionine, lysed and extracts analysed by SDS-PAGE as described by Laemmli (1970). To detect [^{35}S]methionine-labelled proteins the gels were dried under vacuum and exposed to Agfa X-ray films.

Test for kanamycin resistance

E. coli C600 cells (Herrmann *et al.*, 1980), transformed with plasmids of interest, were selected by ampicillin (pBR322 derivatives) or tetracycline (pJKK3-1 derivatives) and transferred with toothpicks onto agar plates containing different concentrations of Km and incubated for 16 h at 37°C.

NPT II-ndPAGE assay

For the preparation of cell extracts analysed in the assay, exponentially growing *E. coli* cells (20 ml) were harvested at a density of 4 x 10^8 cells/ml, spun down and resuspended in 0.5 ml 10 mM Tris-HCl, 10 mM $MgCl_2$, 25 mM NH_4Cl, 0.6 mM β-mercaptoethanol pH 7.4 and sonicated to open the cells. Aliquots of 20 µl of these crude cell extracts were separated by non-denaturing PAGE (10%) and the position of enzymatically active NPT II-like proteins in the gel was determined by *in situ* phosphorylation of Km using [γ-^{32}P]ATP as substrate. The assay was performed as described (Reiss *et al.*, 1984).

In vitro assay of NPT II activity

The NPT II *in vitro* assay was performed essentially as described (Haas and Dowding, 1975) except that toluene-treated *E. coli* cells (McKenney *et al.*, 1981) were used instead of crude *E. coli* cell extracts. The unit of enzymatic activity was defined to be the amount of NPT II enzyme phosphorylating 1 pmol of Km in 30 min at 37°C in that test.

Acknowledgements

We would like to acknowledge H. Weiher for constructive discussion, H. Will and E. Beck for their stimulating interest and V. Bosch for critically reading the manuscript. This work was supported by grants from the Deutsche Forschungsgemeinschaft (Forschergruppe Genexpression) and the Fond der Chemischen Industrie. R. Sprengel is a recipient of a fellowship from the Fritz Thyssen Stiftung.

References

Backman,K., Ptashne,M. and Gilbert,W. (1976) *Proc. Natl. Acad. Sci. USA*, **73**, 4174-4178.

Beck,E., Ludwig,G., Auerswald,E.A., Reiss,B. and Schaller,H. (1982) *Gene*, **19**, 327-336.

Bevan,M.W., Flavell,R.B. and Chilton,M.-D. (1983) *Nature*, **304**, 184-187.

Bolivar,F. (1978) *Gene*, **4**, 121-136.

Colbére-Garapin,F., Horodniceanu,F., Kourilsky,P. and Garapin,A.C. (1982) *J. Mol. Biol.*, **150**, 1-14.

Garoff,H. and Ansorge,W. (1981) *Anal. Biochem.*, **115**, 454-457.

Haas,M.J. and Dowding,J.E. (1975) *Methods Enzymol.*, **43**, 611-627.

Herrera-Estrella,L., De Block,M., Messens,E., Hernalsteens,J.-P., Van Montagu,M. and Schell,J. (1983) *EMBO J.*, **2**, 987-995.

Herrmann,R., Neugebauer,K., Pirkl,E., Zentgraf,H. and Schaller,H. (1980) *Mol. Gen. Genet.*, **177**, 231-241.

Hirth,K.-P., Edwards,C.A. and Firtel,E.A. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 7356-7360.

Kreft,J., Berger,H,. Härtlein,M., Müller,B., Weidinger,P., Goebel,W. (1983) *J. Bacteriol.*, **155**, 681-689.

Kurz,C., Forss,S., Küpper,H., Strohmaier,K. and Schaller,H. (1981) *Nucleic Acids Res.*, **9**, 1919-1931.

Laemmli,U.K. (1970) *Nature*, **227**, 680-685.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning, A Laboratory Manual*, published by Cold Spring Harbor Laboratory Press, NY.

Maxam,A.M. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499-560.

McKenney,K., Shimatake,H., Court,D., Schmeissner,U., Brady,C. and Rosenberg,M. (1981) in Chirikjian,J.G. and Papas,T.S. (eds.), *Gene Amplification and Analysis. 2. Analysis of Nucleic Acids by Enzymatic Methods*, Elsevier-North Holland, p. 383-415.

Müller-Hill,B. and Kania,J. (1974) *Nature*, **249**, 561-563.

Neugebauer,K., Sprengel,R. and Schaller,H. (1981) *Nucleic Acids Res.*, **9**, 2577-2588.

Prentki,G., Karch,F., Iida,S. and Meyer,J. (1981) *Gene*, **14**, 189-299.

Rao,N. and Rogers,S.G. (1979) *Gene*, **7**, 79-82.

Reiss,B. (1982) Ph.D. Thesis, University of Heidelberg.

Reiss,B., Sprengel,R., Will,H. and Schaller,H. (1984) *Gene*, **30**, 217-223.

Rüther,U. and Müller-Hill,B. (1983) *EMBO J.*, **2**, 1791-1794.

Sancar,A., Hack,A.M. and Rupp,W.D. (1979) *J. Bacteriol.*, **137**, 692-693.

Shimkets,L.J., Gill,R.E. and Kaiser,D. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 1406-1410.

Shine,J. and Dalgarno,L. (1974) *Proc. Natl. Acad. Sci. USA*, **71**, 1342-1346.

Southern,P.J. and Berg,P. (1982) *J. Mol. Appl. Genet.*, **1**, 327-341.

Sprengel,R. (1983) Ph.D. Thesis, University of Heidelberg.

Stormo,G.D., Schneider,T.D. and Gold,L. (1982) *Nucleic Acids Res.*, **10**, 2971-2996.

Suttcliffe,I.G. (1978) *Cold Spring Harbor Symp. Quant. Biol.*, **43**, 77-90.

Tooze,J. (1981) *Molecular Biology of Tumor Viruses*, Second Edition Part 2/Revised, published by Cold Spring Harbor Laboratory Press, NY.

Weiher,H. (1982) Ph.D. Thesis, University of Heidelberg.

Weiher,H. and Schaller,H. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 1408-1412.

Zabeau,M. and Stanley,K.K. (1982) *EMBO J.*, **1**, 1217-1224.

Received on 16 October 1984