Both RNA level and translation efficiency are reduced by anti-sense RNA in transgenic tobacco

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ABSTRACT

The effect of anti-sense RNA on the expression of the bialaphos resistance (*bar*) gene which encodes phosphinotricin acetyl transferase (PAT), was analysed in tobacco. Transient expression studies revealed that an anti-*bar* RNA with sequence complementarity to the complete *bar* coding region, inhibits PAT synthesis. To quantify the phenomenon, SR1 tobacco cells were transformed twice to introduce first a hybrid *bar* gene with a reporter gene and in a second instance an anti-*bar* gene. A first cycle transformant and a double transformant derived herefrom in which PAT synthesis was reduced to only 8%, were studied in detail. The interference of the anti-sense gene with the expression of the *bar* gene is manifested at least two levels. First, the *bar* mRNA steady state level is significantly reduced relative to the parental whereas the transcript level of the reporter gene is unchanged. Comparison of *bar* mRNA levels in total and single stranded (ss) RNA preparations demonstrated that little if any stably base-pairing *bar* and anti-*bar* RNA accumulates. Secondly, a three fold reduction of PAT synthesis per *bar* mRNA is observed. This supposes that because of unstable interactions with the complementary anti-*bar* RNA either a substantial part of the *bar* mRNA detected does not enter the cytoplasm and/or that in the cytoplasm the *bar* mRNA is less efficiently translated. It is not clear if or how the reduced *bar* mRNA level is related to such unstable interactions.

INTRODUCTION

Examples of gene regulation by anti-sense transcripts have been described in both procaryotic and eucaryotic systems (1−4). The regulatory effect is believed to evolve from base-pairing between the sense and anti-sense RNA strands by which the messenger is blocked in its expression pathway. In eucaryotes such interaction could occur in the nucleoplasm but also at other locations. The mechanisms suggested suppose that double stranded nuclear RNA is arrested in that organelle whereas double stranded cytoplasmic RNA would interact less efficiently with the translational apparatus or would have an increased turn over. Evidence supporting these different models was obtained by introduction of anti-sense genes and/or transcripts in various *in vivo* and *in vitro* systems (5−7). The demonstration of these regulatory processes is in sharp contrast with the absence of reports describing examples of naturally occurring anti-sense regulation in eucaryotes. However, an RNA duplex unwinding activity has been identified in *Xenopus* oocytes (8), a dsRNAse activity has been found in mouse oocytes (9) and overlapping eucaryotic transcription units have been described in *Drosophila* and mouse (10, 11).

In this report we confirm the feasibility of the anti-sense approach in transgenic tobacco plants (12−14) and study the underlying mechanism. The gene under study is the hybrid P_{TR2}·*bar* gene (*bar*, bialaphos resistance, 15) which in *Nicotiana tabacum* cv. Petite Havana SR1 expresses phosphinothricin acetyl transferase (PAT) up to 0.2% of the soluble

cellular protein fraction (Denecke *et al.*, in preparation). The gene is not essential to the cell and is well expressed in leaf protoplasts. These aspects together with the sensitive PAT bioassays and the abundant steady state *bar* mRNA level make this gene a suitable candidate to identify parameters important in anti-sense gene regulation. The effect of anti-sense RNA on gene expression was studied in tobacco leaf protoplasts since it permits to quantitate RNA levels as well as to measure protein synthesis either by *in vivo* protein labeling or by transient '*de novo*' synthesis. We demonstrate that expression of anti-sense *bar* mRNA reduces the *bar* mRNA steady state level and protein synthesis per *bar* messenger. Possible mechanisms underlying this phenomenon are discussed.

MATERIALS AND METHODS
Nomenclature
Promoter cassettes of plant genes are notated as P_x. 'x' refers to the name of the promoter or the gene from which the promoter is derived. Transgenic *Nicotiana tabacum* cv. Petite Havana SR1 are notated as SR1(T-Y), and in the case of double transformants as SR1(T-Y,T-Z). T-Y and T-Z refer to the T-DNA construction used, named pY and pZ respectively.
DNA manipulations
DNA manipulations were performed essentially as described in (16). pGSC1 is a derivative of pGSFR280 (17). The 570 bp BAMH1 fragment containing the *bar* coding sequence was ligated in inverted orientation in the large BAMH1 fragment of pGSFR280. pGSDE501 and pGEMbar were a gift of J.Denecke, pGSDE501 is a pUC18 (18) derivative carrying in the polylinker the $P_{35S}cat3'ocs$ and the $P_{TR2'}bar3'g7$ genes in a direct orientation. The *cat* sequence is derived from pBR325 (19). pGEMbar is a pGEM2 (Promega Biotech) derivative carrying in the BAMH1 site the 570 bp BAMH1 fragment of pGSFR280 (17) containing the *bar* coding sequence. pGEMhpt is a pGEM2 derivative carrying in the HindIII site the 1 kbp SmaI ScaI fragment of the *hpt* coding sequence (20).
Tissue culture
Leafdisc transformation and protoplast preparation of *Nicotiana tabacum* cv. Petite Havana SR1 (21) was carried out essentially as described by (17). SR1(T-GSFR166) regenerants were selected by their ability to grow on phosphinothricin (PPT, 22) and were a gift of G. Angenon. T-DNA of pGSC1 was introduced into SR1 and SR1(T-GSFR166) and transformants were identified by their kanamycin resistant phenotype.
Electroporation
Electroporation of SR1 and SR1(T-GSC1) protoplasts was carried out as described by Denecke *et al.* (in preparation). Batches of 10^6 protoplasts in 0.3 ml buffer (0.4 M sucrose, 4 mM $CaCl_2$, 80 mM KCl, 10 mM Hepes, pH 7.2) were electroporated in the presence of 10 μg pGSDE501. 8 batches were pooled and divided over 8 vials to minimize effects of variability in electroporation efficiency. At 30 minute intervals samples were frozen to $-70°C$ and stored until further processing.
Enzyme assays
Soluble proteins of frozen cells were extracted according to (17). 3.5 μg of protein was used for both PAT assay and CAT assay. Reactions were done in the presence of excess of substrate. Pat assays were carried out as described by (17). CAT assays were performed as described by (23). 8 μl of all reactions were spotted on a silicagel t.l.c. plate for separation. After chromatography the t.l.c. plates were autoradiographed and films were scanned (LKB ULTROSCAN 2202). The maximal variation between the totals of the lanes of SR1 PAT was 12%, SR1 CAT 8%, SR1(T-GSC1) PAT 11%, SR1(T-GSC1) CAT

7%. The value of ^{14}C-labeled PPT for each sample was divided by the CAT activity value of the same protein sample. The final error is estimated to be less than 20%.

in vivo PAT labeling and immuno-detection
10^5 protoplasts in 100 μl medA (see tissue culture) were incubated for discrete time periods in the presence of 20 μCi ^{14}C-labeled amino acids (Amersham, cfb. 152) at 23°C with low light intensity. After incubation soluble protein was extracted (see enzyme assays). 2−5 μl samples were taken for protein concentration determination (Biorad assay), total label counting and TCA precipitation to determine the incorporation efficiency. PAT was immuno-precipitated from 80 μg protein extract with polyclonal antibody to PAT (17) essentially as described by van (24). Total sample was separated on a denaturing polyacrylamide gel. The gel was dried, autoradiographed and scanned. The intensity of the bands was related to the relative synthesis rate by including a dilution series of ^{14}C-labeled PAT.

RNA manipulations
RNA was extracted from protoplasts essentially as described by (25) and (26). Single stranded RNA was specifically precipitated by an overnight incubation of the nucleic acids samples at 4°C in 2M LiCl. Total RNA was obtained by precipitation at 4°C in 4M LiCl. SP6 and T7 RNA syntheses were performed to obtain cold transcripts of fragments of the *cat*, *bar*, *anti-bar* and *hpt* coding sequences of about 650 nucleotides length using pGEMbar and pGEMhpt. RNA samples were diluted to 1, 2.5, 5, 10, 20, 40 and 80 pg. 1 μg total SR1 RNA was added to each dilution, samples were glyoxylated and applied to a slot blot apparatus. Three times 1 μg of the RNAs to be analysed plus a negative control were applied to the same filter. After baking, filters were boiled for 5′ in 20 mM Tris-HCl pH 8. Filters were probed with ^{32}P-UTP (Amersham) labeled SP6 and T7 transcripts complementary to the cold transcripts mentioned. Autoradiograms were scanned. RNA abundances were determined with the aid of calibration curves obtained with dilution series of the SP6 and T7 transcripts. Due to minor differences in sequence between *in vitro* synthesized RNA and mRNAs all values are approximately 10% underestimated. Northern blots and hybridisations were carried out essentially as described by (25).

RESULTS
The anti-bar gene interferes with transient bar expression
The *bar* coding sequence present on a 570 bp BamHI fragment on plant vector pGSFR280 (17) was inverted in orientation relative to the cauliflower mosaic virus (CaMV) 35S promoter (27), generating pGSC1 (figure 1). The anti-*bar* gene was stably introduced into SR1 by T-DNA transfer, generating SR1(T-GSC1). One to five days after isolation, leaf protoplasts of SR1(T-GSC1) typically express anti-*bar* transcript at an abundance of 2.10^{-5} relative to total RNA. The transcript is polyadenylated and has a length of 700 to 750 nucleotides (not shown), which is in accordance with the distance from transcription start to the 3′ end and a poly(A) tail.

To verify whether the anti-*bar* transcript can interfere with expression of the *bar* gene, an inhibition assay was developed. Construction pGSDE501, which contains the hybrid $P_{TR2'}bar$ and $P_{35S}cat$ marker genes (*cat*, chloramphenicol acetyl transferase, 19; figure 1) was transiently expressed in SR1 and SR1(T-GSC1) protoplasts. The CaMV 35S promoter is approximately equally active in all leaf protoplasts (Jefferson *et al.*, in preparation). This implies that anti-*bar* mRNA is synthesized by all SR1(T-GSC1) cells and that interference with expression of a transiently introduced *bar* gene can occur in
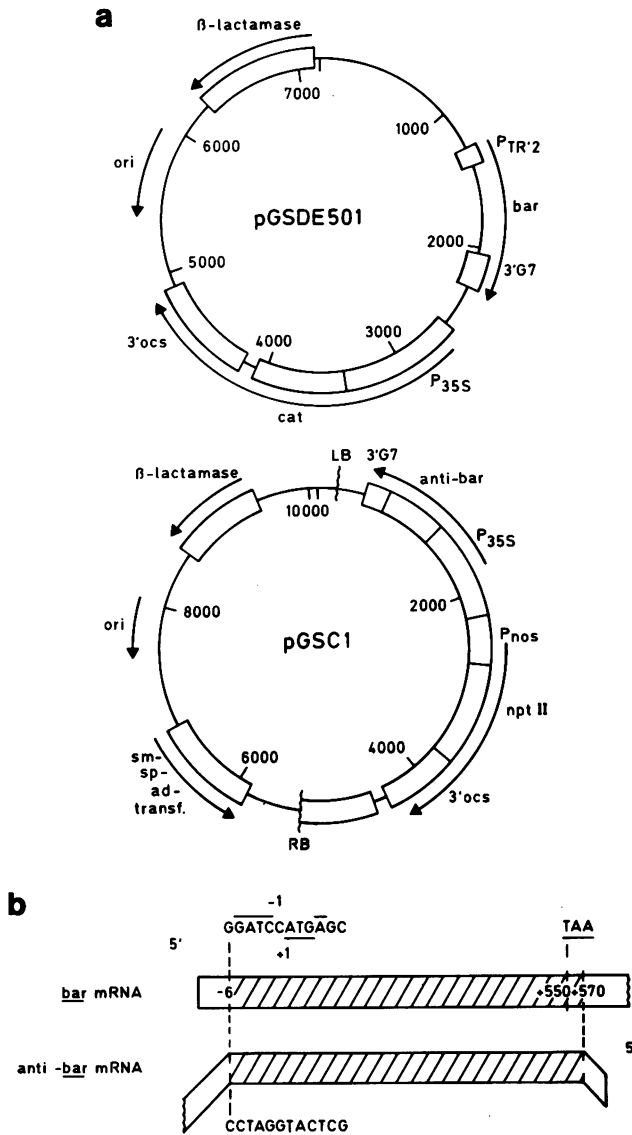
Figure 1a. Schematic presentation of pGSC1 and pGSDE501. LB and RB stand for left and right T-DNA border. **1b.** The anti-*bar* transcript is complementary to 576 nucleotides of the pGSDE501 *bar* transcript. Translation start and stop codons of the *bar* coding sequence are indicated. Numbering refers to the nucleotide position relative to the translation start codon of the mRNA (De Block *et al.* 1987). Overlined nucleotides indicate the differences between TR2′ transcripts of pGSDE501 and pGSFR166 (see figure 3a).

principle in all SR1(T-GSC1) cells. The amount of plasmid specific transcript synthesized is a function of the DNA uptake and the transcriptional activity of the promoters located on the plasmid in a given cell. Because of this, dramatic differences in *bar* mRNA level
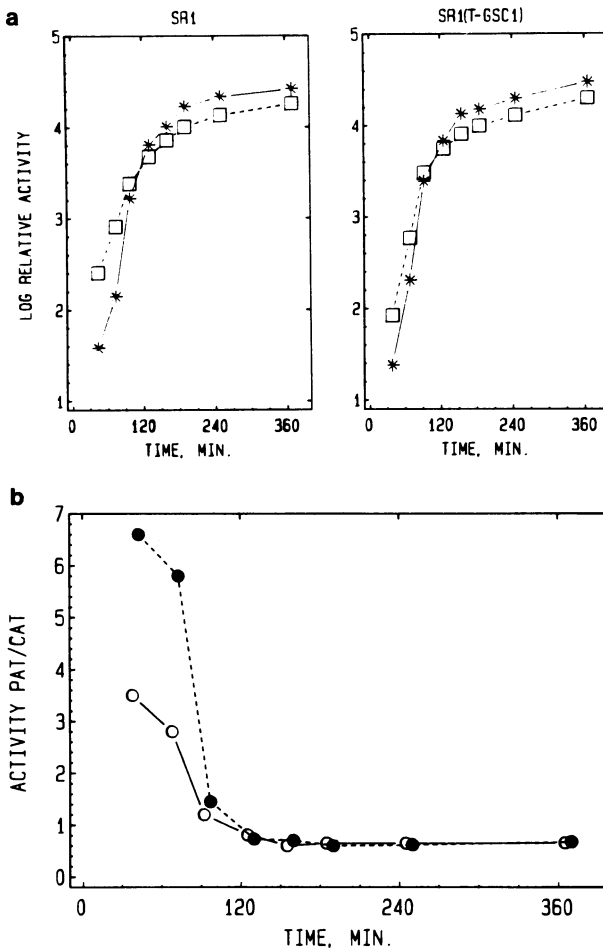
Figure 2a. Relative PAT and CAT enzyme activity in SR1 and SR1(T-GSC1) protoplasts electroporated with pGSDE501 as measured by densitometric scanning of autoradiograms. One of two experiments is shown. Asterixes represent CAT values, open boxes PAT values **2b**. PAT activity relative to CAT activity. Open circles: SR1 (T-GSC1), closed circles: SR1. For calculations see Materials and Methods.

can be expected between individual cells. The extent to which the anti-*bar* mRNA interferes with expression of the *bar* gene in SR1(T-GSC1) cells will, therefore, differ per cell. The $P_{35S}cat$ gene present on pDE501 is expressed in all successfully electroporated cells. Therefore, the $P_{35S}cat$ gene can be used as an internal standard to compare the electroporation efficiency of the two cell lines. By comparing transient PAT expression in SR1 and SR1(T-GSC1) and correcting for electroporation efficiency, a global reduced rate of PAT synthesis may be detectable in the anti-sense strain before maximal *bar* mRNA steady state levels have been reached. The half life of PAT in the cell is over 40 hours (Cornelissen and Vandewiele, in preparation) and exceeds the time span of the experiment. Therefore, the PAT detected represents nearly all PAT synthesized.
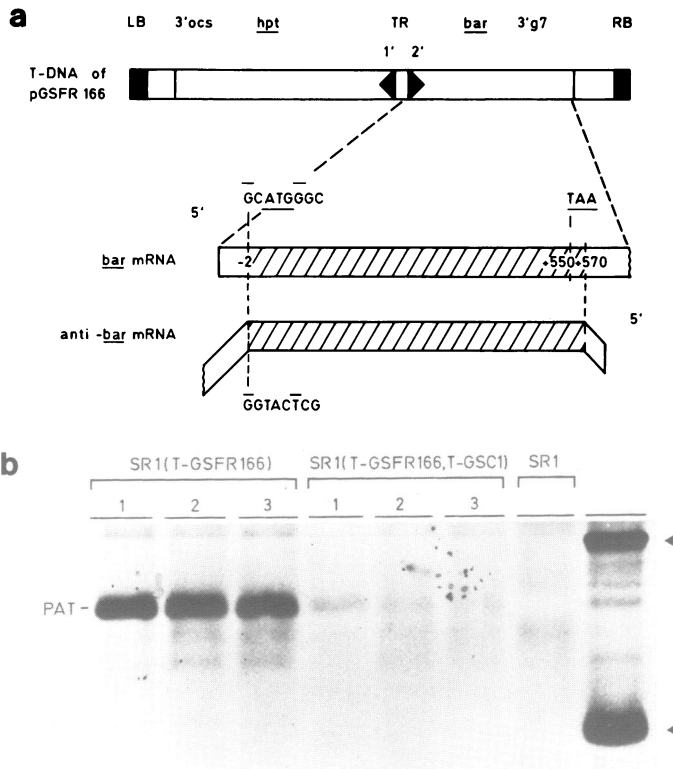
Figure 3a. Schematic presentation of the T-DNA of pGSFR166. The anti-bar transcript is complementary to 570 nucleotides of the *bar* transcript. Single mismatches are overlined. Numbering is relative to the translation start codon of the *bar* mRNA. **3b.** Comparison of the rate of PAT synthesis in SR1(T-GSFR166) and SR1 (T-GSFR166,T-GSC1) protoplasts during the first 72 hours after isolation. 1, 2 and 3 refer to the day on which the 20 hours labeling was carried out. Arrows indicate the 30 and 14.3 kd marker proteins. A constant rate of synthesis during three days was seen in three independent experiments.

PAT and CAT synthesis in SR1 and SR1(T-GSC1) was measured at 30 minutes intervals during the first six hours after electroporation with pGSDE501 (figure 2a). The reduced PAT/CAT activity in SR1(T-GSC1) relative to SR1 at the first three time points (figure 2b) implies that the anti-*bar* mRNA interferes with the expression of the *bar* gene and thus behaves as a functional anti-sense messenger. As time proceeds the retardation of PAT quickly diminishes, probably reflecting titration of anti-sense transcript by *bar* mRNA in cells responsible for the bulk of PAT synthesis. The higher ratio of PAT versus CAT enzyme in both cell lines at t = 30, 60 and 90 minutes may be explained by differences in transcript and coding region length, because of which PAT appears in the cytoplasm earlier.

In transgenic tobacco the anti-bar gene affects the PAT level
The interaction between the *bar* and anti-*bar* genes was studied in stably transformed plants to obtain insight in the mechanism of inhibition. A T-DNA construction carrying the *bar* and *hpt* (hygromycin phosphotransferase, 20) coding sequences under the control of the TR dual promoter (28), was introduced in SR1, generating SR1(T-GSFR166) (Angenon

TABLE 1   Relative PAT synthesis in SR1 (T-GSFR166) derivates carrying the anti-bar gene

Double transformant	Copy number anti-bar gene	% PAT synthesis
1	1	78
2	2	3
3	2	12
4	1	20
5	1	30

PAT synthesis was determined by *in vivo* protein labelling as described in Material and Methods and is given as percentage relative to SR1(T-GSFR166). SR1(T-GSFR166,T-GSC1) is not included.

et al., in preparation). The $P_{TR2'}$·*bar* genes in pGSFR166 and pGSDE501 differ by only 5 nucleotides (figures 1b and 3a). A transformant carrying two T-DNA inserts in its genome and expressing both phenotypes (not shown) was taken for further study. Via a second transformation cycle the T-DNA of pGSC1, which specifies a functional anti-*bar* RNA (see above), was introduced into SR1(T-GSFR166). Six independent regenerants were studied further. In these double transformants Southern analysis could not reveal any modification of the pGSFR166 T-DNA due to the second transformation cycle (not shown). A screening of the six independent regenerants yielded two individuals which displayed in leaf tissue an approximately ten-fold lower PAT activity (17) relative to SR1(T-GSFR166) (not shown). One of these two plants, named SR1(T-GSFR166,T-GSC1), was taken for further study. This double transformant carries two copies of the anti-*bar* gene.

Leaf protoplasts were prepared from SR1(T-GSFR166,T-GSC1), as well as from SR1(T-GSFR166), and incubated at low light intensity for a period of one, two or three days. For each time point the last 20 hours of incubation were in the presence of ^{14}C-labeled amino acids to determine *de novo* protein synthesis. After incubation the cells were lysed, PAT was immuno-precipitated and visualized by autoradiography after gel electroforeses. Figure 3b shows that during the first 72 hours after protoplast preparation PAT synthesis is constant for both cell lines. Therefore, the translatable *bar* transcript levels in these cell pools will also be rather constant over this period. Thus, it is possible to relate the RNA steady state level to the amount of labeled translational product formed. Scanning of the autoradiograms shown in figure 3b demonstrates that the SR1(T-GSFR166,T-GSC1) cells produce on average 13 fold less PAT than the parental. This indicates that the anti-*bar* transcript heavily interferes with the expression pathway of the *bar* gene.
Noteworthy is that when the other five double transformants of the initial screening were subjected to this type of assay, they all displayed a reduced PAT synthesis (table 1). Such reduction was not appreciated for all regenerants in the first screening, probably because of variability in the leaves of the different transformants and the lack of an internal reference.
The effect of anti-sense RNA on expression is manifested at at least two levels
Leaf protoplasts of SR1(T-GSFR166) and SR1(T-GSFR166,T-GSC1) were incubated for 48 hours at low light intensity. Both total and single stranded RNA were extracted in order to determine if *bar*−anti-*bar* duplex RNA accumulates. In the hours directly before and after the RNA extractions a 20 hours *in vivo* protein labeling and PAT immuno-precipitation

TABLE 2	Transcript abundances and relative rate of PAT synthesis in leaf protoplasts	
	SR1(T-GSFR166)	SR1(T-GSFR166,T-GSC1)
bar-mRNA	45	11/11*
anti-bar-mRNA	0	7
hpt-mRNA	30	27
relative PAT synthesis	13	1

Values are in pg per μg total RNA and are the average of three measurements. Asterix refers to *bar* mRNA in total RNA preparations. Method of quantization is described in Materials and Methods. Repetition of the experiment gave comparable results.

was carried out with samples of both cell lines in order to relate the *bar* mRNA levels to the relative rates of PAT synthesis. The abundance of *bar* mRNA, *hpt* mRNA and anti-*bar* mRNA of both samples was determined by slot-blot analysis. Table 2 shows that the *hpt* transcript abundance in both cell lines is comparable, differing only by 20 %. Since the transcriptional activities of the *hpt* and *bar* genes are linked by the TR dual promoter (28, figure 3a), the comparable *hpt* RNA levels indicate that the SR1(T-GSFR166,T-GSC1) cells display a bonafide expression of the genes located on the T-DNA of pGSFR166. Interestingly, the ss *bar* mRNA level in SR1(T-GSFR166,T-GSC1) is 4 fold lower than in SR1(T-GSFR166) (table 2, figure 4). In view of the comparable *hpt* levels, the nature of the TR dual promoter and the demonstrated inhibitory effect of the anti-*bar* messenger (figure 2), the more likely explanation for the reduced *bar* mRNA level is that interaction between the complementary RNAs results in a reduced *bar* mRNA steady state level. Comparison of the abundance of *bar* mRNA in SR1(T-GSFR166,T-GSC1) protoplasts in total and single stranded RNA preparations (table 2) does not reveal a significant difference which implies that base-paired *bar* and anti-*bar* RNAs do not accumulate to high levels. Control experiments, in which protoplasts of SR1(T-GSFR166) and SR1(T-GSC1) were mixed and used for total and single stranded RNA preparation, indicated that during the extraction there is no detectable formation of duplex *bar* and anti-*bar* RNA nor specific degradation of either RNA species (not shown).

However, the 4 fold reduced *bar* mRNA level in the double transformant does not correspond with the 13 fold lower amount of synthesized PAT. If a linear relation between the levels of a given mRNA and the production of translational product is assumed, then the reduction in the accumulation of PAT is 3 fold more than expected from the mRNA levels only. This implies that the translatability of the steady state *bar* mRNA pool has decreased in cells expressing the anti-*bar* mRNA.

DISCUSSION
The effect of anti-sense RNA on gene expression was studied in tobacco. As a model system the *bar* gene was chosen because its gene product is not essential to the cell implying that
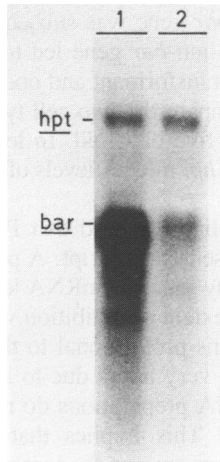
Figure 4. Northern blot analysis of ss RNA extracted from protoplasts of SR1(T-GSFR166) (lane 1), SR1(T-GSFR166,T-GSC1) (lane 2). The filter was hybridised with probes complementary to the *bar* and *hpt* transcripts.

changes in PAT level will not evoke any stress responses. A high level of anti-*bar* mRNA was obtained in tobacco by expression of a hybrid gene containing the *bar* coding region in inverted orientation under the control of the CaMV 35S promoter. The 35S promoter is transcriptionally active in all leaf protoplasts. Therefore, leaf protoplasts carrying an anti-sense gene under control of 35S, provide an ideal system to quantify the effects of anti-sense RNA on expression.

The anti-*bar* gene interferes with the expression pathway of the *bar* gene both in transient expression and in stable transformants. Transient expression of the *bar* gene in protoplasts from both SR1 and a SR1 strain expressing the anti-*bar* transcript revealed that in the first two hours after introduction, PAT synthesis is specifically retarded in cells expressing the anti-*bar* transcript. The effect represents a summation of PAT levels in all cells. The DNA uptake and expression vary per electroporated cell, leading to different ratios between sense and anti-sense RNA. The transient effect of inhibition can be explained by the excess of anti-sense transcript in the initial phase of *bar* mRNA production. Accumulation of the *bar* mRNA to steady state level in the cells responsible for the bulk of PAT will lead in these cells to titration of the anti-sense transcript. This results in a loss of the inhibition phenotype of the cell pool. The extent and the duration of inhibition will be determined by a complex of factors including the RNA synthesis, the half life, the sequence complementarity and the RNA secondary structure. We did not look at the complete expression curve for both cell lines. At a late stage when the cells produce less plasmid specific transcripts, a relatively reduced PAT synthesis in SR1(T-GSC1) can again be expected. However, due to the high stability of PAT it will be impossible to detect this decrease in this type of assay.

To obtain insight into the mechanism by which anti-sense RNA interferes with expression, the interaction was studied in stably transformed leaf protoplasts. The expression of the *bar* gene was followed both at mRNA and protein synthesis level in the presence or absence of the anti-*bar* gene. Surprisingly, the inhibition results from at least two phenomena. First, the *bar* mRNA pool is decreased significantly in size and secondly, the protein synthesis per *bar* mRNA is strongly reduced. In practice, the *bar* gene and a *hpt* reporter gene were

introduced into SR1 and the anti-*bar* gene was subsequently introduced in the resulting transformant. The presence of the anti-*bar* gene led to a reduction of PAT synthesis in all the plants analysed. The single transformant and one of the double transformants were studied in detail. To be able to compare the two cell types, all experiments were carried out with leaf protoplasts of one to five days old. In leaf protoplasts of this age the rate of PAT synthesis is constant and the *hpt* mRNA levels of the single and double transformant are comparable.

Protein analysis of the two cell lines showed that PAT synthesis is 13 fold lower in leaf protoplasts expressing the anti-sense transcript. A partial explanation for this decrease is provided by the 4 fold lower steady state *bar* mRNA level in these protoplasts. Repetition of the experiment showed that the extent of inhibition varies. The difference in reduction of PAT synthesis, however, remains proportional to the reduction in *bar* mRNA level. The reduced *bar* mRNA levels are very likely due to interactions between *bar* and anti-*bar* transcripts. However, total RNA preparations do not contain substantially more *bar* RNA than ss RNA preparations. This implies that if stable duplexes between the complementary RNAs are formed they are rapidly degraded. Accumulation of duplex RNA seems to vary depending on the experimental system. For example, Kim and Wold (6) have shown that in mouse cells duplex mRNA is arrested in the nucleus and accumulates to detectable levels. Instead, Crowley *et al.* (6) did not detect ds RNA in Dictyostelium and concluded that duplex RNA possibly is trapped in the nucleus and is rapidly degraded. It is clear from these different data that accumulation of duplex RNA as well as the level of anti-sense RNA detected are not necessarily related to the level of expression inhibition.

Interestingly, the protein synthesis per *bar* mRNA in the double transformant is reduced to approximately 30 % relative to the parental. This can be interpreted mechanistically in two ways. If a major part of the ss *bar* mRNA detected represents mRNA that has not entered the cytoplasm, it would imply that the unstable interactions between the complementary RNAs greatly retard the RNA transport to the cytoplasm. However, Kim and Wold (6) and Crowley *et al.* (4) assigned in their experiments single stranded RNA only to the cytoplasm. Thus, it is more likely that the ss RNA detected represents cytoplasmic RNA and that in the cytoplasm the anti-sense transcript hinders the translation of the *bar* mRNA, probably by unstable base-pairing of the complementary regions. We do not know if the interaction which leads to a reduced protein synthesis is also responsible for the reduced steady state mRNA level perhaps by destabilising the mRNA. Experiments are in progress to localize the *bar* transcripts and to identify the interactions of the *bar* transcripts with both the translational apparatus and the anti-*bar* RNA.

Insight in requirements for maximal interference is essential to successfully use the anti-sense approach in studies aimed at functional analysis of an uncharacterized gene product. We obtained up to 97% inhibition by expression of a transcript species complementary to a large region of the *bar* mRNA, which is consistent with results from other laboratories (6, 12). Requirements for an anti-sense gene with a maximal inhibitory effect will depend on the steps which are crucial in the mechanism of inhibition. It can be expected that both the site(s) of interaction and the specific sequence of the RNA molecules are fundamental. However, RNA duplex formation and the preceeding steps may prove as complex as described for the procaryotic ColE1 system (29, 30), where determinants of the binding efficiency include secondary structure constraints and inter-molecular base pairing strength at strategic places within the secondary structure.

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