# Short Technical Reports

## Novel Vector for Generating RNAs with Defined 3′ Ends and Its Use in Antiviral Strategies

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**ABSTRACT**

*A novel transcription system was constructed that allows trimming of 3¢ termini of RNA transcripts in* E. coli *by endogenous RNase P. Here, the sequence of tRNA$^{Ser}$ from* E. coli *fused downstream of the target sequence directs posttranscriptional cleavage 3¢ of the target sequence. As a first-target MNV11(+), a self-replicating RNA from the Qb system was subjected to transcription in vivo. Northern blotting experiments revealed that the primary transcript was indeed successfully processed to an RNA of expected length. The RNA released proved to function as an active template for Qb replicase. Moreover,* E. coli *cells producing these short-chain replicator molecules no longer supported multiplication of Qb phages upon infection. Since the novel transcript-trimming system utilizes the endogenous RNase P activity and does not depend on any particular 3′-terminal RNA sequence of target molecules, it may have wide applications for a number of different targets in prokaryotes. Further applications, including those in eukaryotes, are discussed.*

### INTRODUCTION

In a number of instances, it is particularly important to generate RNA transcripts with fairly or sometimes even very accurate 3¢ ends in vitro and in vivo. In vitro, the use of bacteriophage polymerases is the common method for producing large amounts of RNA. Here, however, primary transcripts exhibit a pronounced heterogeneity concerning their 3′ ends (18,21). To trim the ends of the transcripts, hammerhead sequences have been designed into the transcription cassette (34,35). RNA cleavage is then guided by the hammerhead fold to a well-defined phosphodiester bond in the primary transcript. Since one necessary prerequisite for hammerhead cleavage is the presence of the trinucleotide NUX sequence (N = G, A, U, C; X = C, A, U) immediately 5¢ to the cleavage site (26,30), the trimming method of transcripts is restricted to only 12 from 64 possible 3′-terminal trinucleotide sequences, excluding, e.g., CCA ends of tRNAs and tRNA-like structures.

Here we provide an alternative approach that is sequence-independent. Transcription cassettes are designed with a tRNA sequence just downstream of the target sequence. Primary transcripts then function as substrates for RNase P, which specifically cleaves immediately 5′ of the tRNA sequence. Determinants for the positioning of the cleavage site reside almost exclusively in the tRNA part (8,13,16,17,19,20,32, 33,36), making it a suitable RNA module processing from outside the target sequence. This approach was used to generate MNV11(+) RNAs (2) in *E. coli*. Bacteria producing MNV11(+) RNAs were shown to contain biologically active, self-replicating RNAs, which upon infection with Qβ phage, exhibit a strong antiviral activity.

### MATERIALS AND METHODS

#### Oligonucleotide Synthesis

Oligodeoxyribonucleotides (Table 1) were synthesized using standard phosphoramidite chemistry. Purification included denaturing polyacrylamide gel electrophoresis (PAGE), "crush and soak" elution (27) and ethanol precipitation.

#### Plasmid Constructions

Oligonucleotides Trp1–6 corresponding to positions -19 to -1 of the translocated promoter (trp), the sequence of MNV11(+) and the first 33 nucleotides of tRNA$^{Ser}$ from *E. coli* were ligated (31) into the *Spe*I/*Sal*I-digested vector pDR720 (Pharmacia Biotech, Uppsala, Sweden), yielding vector pDR720-MNV. A point mutation (A3 → G3) in the sequence of tRNA$^{Ser}$ was introduced to generate a *Sma*I restriction site exactly at the beginning of the tRNA sequence. The *Bam*HI/*Eco*RI fragment from M13mp18 (Ser-tRNA-CCA) (29) containing the tRNA$^{Ser}$ sequence was ligated into the *Bam*HI/*Eco*RI double-digested vector pT7-1 (Boehringer Mannheim GmbH, Mannheim, Germany) yielding vector pT7-tRNA. The 1480-bp *Ban*I fragment from pDR720-MNV and the 1400-bp *Ban*I fragment from pT7-tRNA were then ligated to generate vector pAS43. Ligation of the 1480-bp *Ban*I fragment from pDR720-MNV and the 1400-bp *Ban*I fragment from pT7-1 yielded pAS84. To construct pAS43-T, oligonucleotides Term1 and Term2 were ligated into the *Sfu*I/*Nde*I double-digested vector pAS43. Similarly, oligonucleotides Ansens53-1 and Ansens53-2 were ligated into the *Spe*I/*Xma*I double-digested vector pAS43-T, generating pAS53-T. For the construction of pAS43-T7 oligonucleotides, T7P1 and T7P2 were ligated into the *Hin*dIII/*Spe*I double-digested vector pAS43-T, replacing the trp with a T7 promoter. All constructs were confirmed by standard sequencing procedures (28).

#### Northern Blot Analysis of Total Bacterial RNA

*E. coli* strain XL1-Blue transformed with plasmids as indicated was grown at 37°C in M9CA medium (27) supplemented with 2 g/L glucose, 0.4 g/L $MgCl_2$•6 $H_2O$, 22 mg/L $CaCl_2$•6 $H_2O$, 50 mg/mL of ampicillin and tryptophan and 3-β-indoleacrylic acid (IAA) as indicated. At optical density $(OD)_{600}$ = 0.2–0.3, chloramphenicol was added to a final concentration of 1 µg/mL. After 10 min of further incubation, cells were collected by centrifugation. Total cellular RNA was extracted according to the protocol of Höfle (10,11) and fractionated by denaturing PAGE. Electro-blotting and hybridization procedures were carried out according to the protocol of the suppliers using Immobilon®-N membranes (Millipore, Eschborn, Germany). To detect MNV11(+) sequences, $^{32}$P-labeled oligonucleotide Trp2 was used as a probe.

#### RNA Growth Curves

RNA replication was monitored by measuring the ethidium bromide (EtdBr) fluorescence (excitation at 514 nm, emission at 600 nm) in a Model LS5B Fluorimeter (Perkin-Elmer, Norwalk,

CT, USA). One hundred-microliter reaction mixtures containing 50 mM Tris-HCl, pH 7.5, 10% glycerol, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 100 mM NaCl, 0.5 mM each NTP, 1 µM Qβ replicase and 15 µM EtdBr were preincubated for 1 min at 30°C. The reaction was initiated by the addition of 10 ng of total cellular RNA. Under these conditions, the detection limit is about $5 \times 10^{-8}$ M MNV11-RNA, and the doubling time is approximately 20 s (G. Strunk, personal communication).

### One-Step Phage Growth Curves

*E. coli* strain JM105 transformed with pAS43-T and pAS53-T, respectively, was grown in M9 medium containing 50 mg/mL ampicillin and tryptophan and IAA as indicated. At $OD_{600}$ = 0.3, cells were infected with Qβ phage using a multiplicity of 0.03. Quantitative adsorption of phage particles to host bacteria was confirmed by counting plaque-forming units (pfu) in the supernatant after infection. Infection was monitored by counting pfu for 70 min after infection using the protocol of Bresch et al. (3).

## RESULTS AND DISCUSSION

### Plasmid Constructions

A novel transcription system was constructed to generate virtually any target RNA sequence with defined 3′ ends in vivo. It is reminiscent of processing of tRNA precursors, since it uses the intrinsic RNase P activity of bacteria cells to cleave primary transcripts accurately between the target sequence and a guide module, which is most simply represented by a tRNA sequence. Figure 1 displays an example of the transcription cassette. Here, the sequence of MNV11(+), a self-replicating variant of the Qβ RNA, is placed between the trp and the $tRNA^{Ser}$ sequences. The trp terminator was placed at the end of the transcription unit. A unique *Spe*I site in the trp sequence and a unique site of the blunt end-generating enzyme *Sma*I (*Xma*I) exactly at the end of the MNV11 sequence were included to easily replace the MNV11

**Table 1. Oligodeoxyribonucleotides Used in the Experiments**

| | |
|---|---|
| Trp1 | 5′-CTAGTTAACTAGTACGCGGGTTCATAGCCTATTCGGCTTT-TAAAGGACCTTTTTCCCTCGCG-3′ |
| Trp2 | 5′-GTAGCTAGCTACGCGAGGGAAAAAGGTCCTTTAAAAGCC-GAATAGGCTATGAACCCGCGTACTAGTTAA-3′ |
| Trp3 | 5′-TAGCTAGCTACGCGAGGTGACCC-3′ |
| Trp4 | 5′-CCCTTCGGGGGGTCACCTCGC-3′ |
| Trp5 | 5′-CCCGAAGGGGGGTGCCCCGGGAGTGTGGCCGAGCGG-TTGAAG-GCACC-3′ |
| Trp6 | 5′-TCGAGGTGCCTTCAACCGCTCGGCCACACTCCCGGGG-CACCC-3′ |
| Term1 | 5′-CGAATCTCTGCGCTTCCGCCAGCCCGCCTAATGAGCGG-GCTTTTTTTTGAACA-3′ |
| Term2 | 5′-TATGTTCAAAAAAAAGCCCGCTCATTAGGCGGGCTGGCG-GAAGCGCAGAGATT-3′ |
| Ansens53-1 | 5′-CTAGTTAACTAGTACGCAGATGCTGTCTTAGACATGCATT-TCAT-3′ |
| Ansens53-2 | 5′-CCGGATGAAATGCATGTCTAAGACAGCATCTGCGTACTA-GTTAA-3′ |
| T7P1 | 5′-AGCTTAATACGACTCACTATAGGGAG-3′ |
| T7P2 | 5′-CTAGCTCCCTATAGTGAGTCGTATTA-3′ |

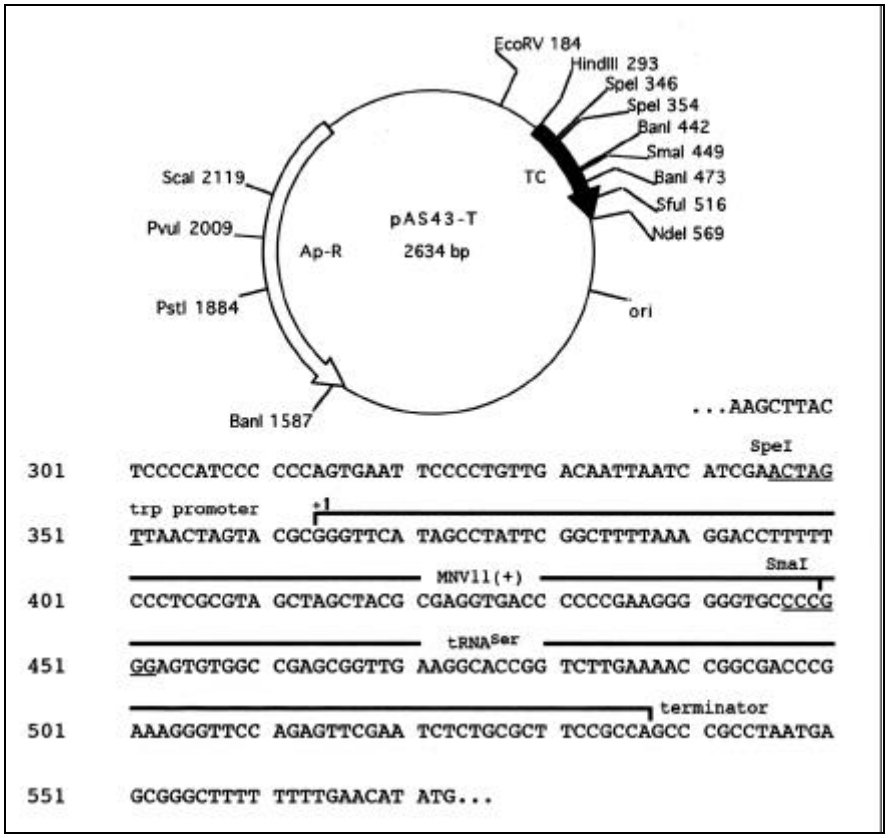


**Figure 1. Plasmid pAS43-T.** Top: Restriction map. Bottom: Sequence of the transcription cassette (TC) between restriction sites of *Hin*dIII and *Nde*I.

target module with, basically, any other sequence. The *Sma*I site was generated by an A3→G3 mutation in the tRNA sequence, maintaining the possibility of base pairing in the acceptor stem. Although this mutation could possibly enhance mischarging of the processed tRNA in vivo (22,23,25), it should not alter the substrate properties for RNase P. On one hand, chemically synthesized double-stranded oligonucleotides with one *Spe*I-compatible end and one blunt end can be ligated. Sequences adjacent to the *Spe*I-compatible end have to include positions -18 to -1 of the trp, which has been removed from the pAS43-T vector. In this way, pAS53-T was constructed containing the sequence of an antisense RNA directed against phage Qβ RNA. Alternatively, *Spe*I-digested polymerase chain reaction (PCR) products can be cloned if one of the primers contains a unique *Spe*I site, part of the trp and the 5′ region of the template. Preferentially, *Pfu* DNA Polymerase (Stratagene, La Jolla, CA, USA) is used to yield a blunt end at the other terminus of the PCR product.

### In Vitro and In Vivo Cleavage of Primary Transcripts

For initial in vitro cleavage studies, vector pAS43-T7 was constructed. Run-off transcripts from the *Nde*I-linearized vector were cleaved in vitro by M1 RNA in a standard cleavage assay (8) revealing products of the expected size (data not shown). In vivo processing reactions were characterized by Northern blot analysis of total cellular RNA (Figure 2) using oligodeoxyribonucleotide Trp2 as a probe. A product of the size of MNV11(+) RNA is visible only in RNA extracts from cells harboring pAS43-T under nonrepressive conditions for the trp. The same RNA preparations contain at least two major side products, one of which appears at higher molecular weight than MNV11(+). It is absent in the total RNA from cells containing pAS84, which lacks the 3′ half of the tRNA and the trp-terminator sequences. Since this product gives no clear signal with oligodeoxyribonucleotide Term2 as a probe (data not shown), this molecular species is presumably not the unprocessed precursor. A strong band at lower molecular weight hybridizes with the probe specific for the 5′ end of MNV11(+) RNA but not with a probe specific for the 3′ end (data not shown). This is most probably caused by a product of premature termination at an internal terminator-like structure rather than a product of degradation.

### Self-Replication of Cleavage Products

The replication of MNV11(+) RNAs critically depends on the correct 3′-end formation. Neither variants missing a single C (14) nor extended variants, e.g., those containing an additional G, are active. Only A extensions seemed to be tolerated to some extent. Therefore, we took self-replication as a criterion for correct RNase P cleavage in vivo. Hence, total cellular RNA from bacteria hosting different vectors was incubated with Qβ replicase (Figure 3), and the EtdBr fluorescence was monitored. In the case of pAS53-T-derived RNA, no increase in fluorescence (i.e., no increase in RNA concentration) was monitored, indicating that no self-replicating RNA was present in total cellular RNA. In contrast, the pAS43-T-derived RNA contains considerable amounts of amplifiable RNA with a length corresponding to the size of MNV11, as has been verified by denaturing PAGE (data not shown).

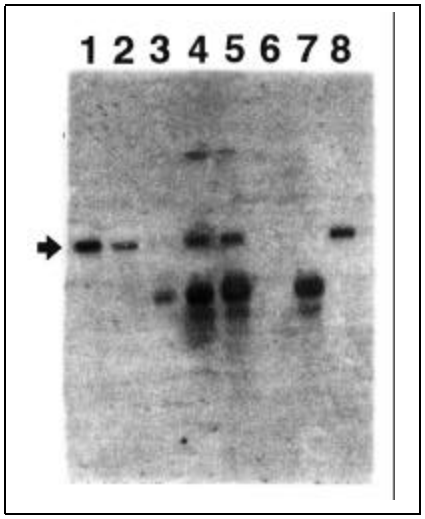


**Figure 2. Northern blot analysis of total RNA.** RNA was isolated from *E. coli* XL1-Blue transformed with different plasmids grown in M9CA media supplemented as indicated. Oligodeoxyribonucleotide Trp2 was used as a probe. The arrow marks the position of MNV11(+). Lanes 1, 2 and 8: 0.2, 0.05 and 0.2 µg MNV-11(+), respectively; lane 3: XL1-Blue/pAS43-T (suppl.: 100 µg/mL tryptophan); lane 4: XL1-Blue/pAS43-T (suppl.: 1 µg/mL tryptophan); lane 5: XL1-Blue/pAS43-T (suppl.: 1 µg/mL tryptophan and 5 µg/mL IAA); lane 6: XL1-Blue/pAS53-T (suppl.: 1 µg/mL tryptophan); and lane 7: XL1-Blue/pAS84 (suppl.: 1 µg/mL tryptophan).

### Antiviral Activity of Self-Replicating RNAs

Finally, the novel transcription system was applied in the context of two RNA-based artificial "immune systems" against bacteriophage infection. First, pAS43-T was used to introduce MNV11(+) RNA into host bacteria. Compared to bacteriophage Qβ RNA, these short-chained RNA species should be potentially good competitors because they (*i*) replicate much faster, (*ii*) exhibit a significantly higher affinity for Qβ replicase (37) and (*iii*) should exclusively interact with replicase, because they do not enclose ribosomal or coat protein-binding sites. As a reference, pAS53-T was utilized to generate antisense RNAs against a region in the replicase cistron of bacteriophage Qβ RNA. There is experimental evidence on the phylogenetically, closely related phage SP that antisense RNA against this region is a potent inhibitor of phage infection (7). One-step phage-growth

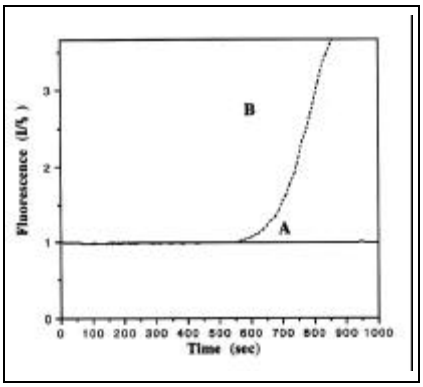


**Figure 3. Growth curves of samples from total cellular RNA of XL1-Blue transformed with pAS53-T (A) and pAS43-T (B).** Cells were grown in M9CA media supplemented with 50 µg/mL ampicillin and 1 µg/mL tryptophan. Total cellular RNA was extracted as described. Replication was initiated by the addition of 10 ng of total cellular RNA. The increase in RNA concentration was monitored by measuring the EtdBr fluorescence.

experiments with bacteria hosting either of the transcription vectors pAS43-T or pAS53-T revealed that the intracellular RNA produced from the transcription cassette had a pronounced effect on the time course of infection by Qβ phage (Figure 4). The antisense RNA introduced by pAS53-T reduced the burst size to 50% of the number obtained with fully repressed host cells. MNV11(+) RNA transcribed from pAS43-T reduced the burst size to ≤1, presumably by competition between the short "replicators" MNV11 and the invading viral RNA for its replicase. In all cases, a quantitative adsorption of phages to the host cells was observed.

### Further Applications of the Transcript-Trimming System

Principally, the transcript-trimming system presented in this paper should work with any 3′-terminal sequence. The information to determine the position of the cleavage site resides completely within the tRNA module. However, care should be taken to avoid tRNAs with determinants, including part of the cleavage site nucleotides and/or base pairing between the flanking target sequence and the tRNA, such as in $tRNA^{His}$ (4,9,16) or $tRNA^{Tyr}Su3$ (16,19,32,33). Since cleavage specificity for a given precursor molecule can be different for RNase P from different organisms (17), it is advisable to use natural tRNA sequences belonging to the organism of concern. It is known that the tertiary folding of the tRNA moiety of a tRNA precursor molecule plays a significant role in the enzyme-substrate interaction (1,15). As in the hammerhead approach (34,35), alternative folding of tRNA segments with upstream elements of the target sequence may occasionally occur and prevent cleavage, as has been shown for yeast

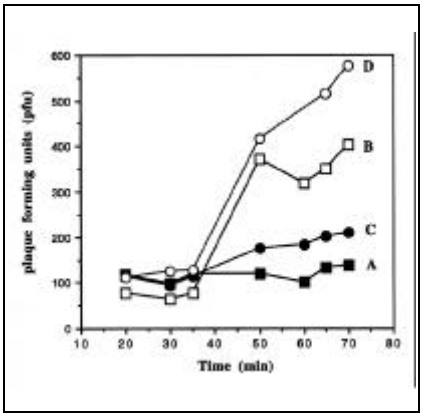


**Figure 4. One-step growth curves of Qβ phage.** Different host cells were grown in M9 medium supplemented with 50 µg/mL ampicillin and 1 µg/mL tryptophan. Host strain JM105/pAS43-T under conditions of nonrepressed (A) and fully repressed (B) trp. Host strain JM105/pAS53-T under conditions of nonrepressed (C) and fully repressed (D) trp. In vivo trancription of antisense RNA from pAS53-T reduces the burst size from 4.6 to 1.9, whereas transcription of MNV11(+) RNA reduces the burst size from 5.1 to approximately 1.

mitochondrial RNase P substrates (12). In addition, endogenous (CCA) nucleotidyltransferases may eventually alter the structure of the 3′ terminus after RNase P cleavage. So far, our experimental results suggest that the method described in this paper is well-suited for applications in *E. coli*. Nevertheless, it may very well work in other prokaryotes and even in eukaryotic cells as well. Comparative studies already indicate that eukaryotic RNase P specificities seem to be fixed absolutely to position +1 (5,6,17). It is, however, not clear if the approach would work readily in eukaryotes using endogenous RNase P. If transcription occurs in the cytoplasm, as in the case of virus-derived vectors, it is uncertain whether nuclear localized RNase P would be accessible. This problem could possibly be avoided if transcription would be from a nuclear transgene. The use of such a system, however, may still be a little cumbersome. Finally, note that processing by RNase P can also be an appropriate method to trim in vitro transcripts. Products processed in this way would exhibit a significant increase in homogeneity, making them better targets for structural analysis, e.g., by nuclear magnetic resonance (NMR) or X-ray crystallography (24).

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# Determination of Transgene Copy Number and Expression Level Using Denaturing Gradient Gel Electrophoresis

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**ABSTRACT**

*Transgenic mice and cell lines are frequently developed to study human disease. Accurate determination of transgene copy number and levels of mRNA are necessary to understand the phenotypic changes observed in these models. Currently, transgene copy number and expression are estimated by Southern blot analysis of genomic DNA and Northern blot analysis of mRNA. We report a novel PCR-based method for determining transgene copy number and levels of transgene expression using competitive PCR between endogenous genomic genes and mutant transgenes followed by denaturing gradient gel electrophoresis (DGGE). We are able to accurately quantify a range of 1–10 copies of transgene incorporated per diploid genome. After reverse-transcribing RNA to cDNA, we are able to quantify levels of transgene mRNA that correlate with biochemical and histological evidence of transgene activity. In conclusion, resolving PCR and reverse transcription-PCR products by DGGE is a rapid and reproducible method that allows for accurate determination of transgene copy number and expression. This technique provides a more complete understanding of transgene effects.*

## INTRODUCTION

Small nucleotide deletions, additions or substitutions can alter gene expression and may be associated with human diseases. One highly sensitive technique used to detect these nucleotide sequence changes is denaturing gradient gel electrophoresis (DGGE) (1,4). In DGGE, DNA is electrophoresed through a polyacrylamide gel in which the concentrations of the denaturants urea and formamide increase linearly. As a double-stranded DNA fragment travels through the gel, it reaches a denaturant concentration that corresponds to its "melting point". At that point, a conformational change occurs that alters the mobility of the fragment. Denaturation occurs under characteristic conditions that reflect not only the length of a DNA fragment but also its sequence. To improve the ability of DGGE to detect small mutations throughout a DNA fragment, one polymerase chain reaction (PCR) primer is synthesized with a GC-rich sequence at its 5′ end to introduce a "GC clamp" with a high melting point into the DNA product. This transforms the amplified fragment into a single melting domain and improves the sensitivity of the technique (1). Thus DNA fragments that differ only by a single base can be distinguished by comparing their migration patterns through denaturing gradient gels (1,4,7,9).

DGGE of a DNA sample that contains equal numbers of two DNA fragments that differ by only a single base, as occurs in many heterozygous genetic diseases, resolves four distinct bands. The two most rapidly migrating bands represent the two homoduplex DNA fragments, whereas the two more slowly migrating DNA fragments represent heteroduplex DNA fragments produced during PCR. Because the generation of homoduplex DNA fragments is based upon the competitive nature of the PCR, we hypothesized that DGGE could be used to determine the relative number of copies of two similar genes in a DNA sample. To test this hypothesis, we analyzed genomic DNA and cDNA from transgenic mice in which expression of a constitutively activated form of the alpha chain of the G protein Gq (Q209L) that differs from wild-type murine $G_{\alpha q}$ by a single base pair (3) was targeted to the thyroid gland by linking Q209L to the rat thyroglobulin gene promoter (TGGQ) (8). In this paper, we describe the use of DGGE to determine the number of copies of the TGGQ transgene that have been integrated into the genome of the transgenic mice. In addition, by using DGGE to analyze cDNA prepared by reverse transcription (RT)-PCR of thyroid RNA from these mice, we have been able to compare levels of transgene-derived Gq mRNA to levels of genomic wild-type Gq mRNA.

## MATERIALS AND METHODS

### Oligonucleotide Primers and Template DNA and RNA

Oligonucleotide primers were designed to amplify a 149-bp region surrounding the $Gln^{209}$ codon of murine $G_{\alpha q}$ (10). One primer of the pair (mdr 2) was synthesized with a 40-base GC-rich extension at the 5′ end (1). The sequence of the GC "clamp" is the following:

5′-CGCCCGCCGCGCCCCGCGCC-CGCCCCGCCGCCCCCGCCCG-3′.

The sequences of the primers are:

Sense: 5′-CAGAATGGTCGATGTAG-GGGGC-3′ (mdr 1)

Antisense: 5′-$(GC)_{40}$CTGACTCCAC-AAGAACTTGATCATATT-3′ (mdr 2)

Genomic DNA was isolated from tail biopsies of TGGQ transgenic mice (8). Total RNA was isolated from the thyroid glands of several mice in each