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Codon context effect in virus translational readthrough

A study in vitro of the determinants of TMV and Mo-MuLV amber suppression

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To assess the role of codon context on the efficiency of eukaryotic suppression of termination codons, we have compared, in a rabbit cell-free translation system, the readthrough efficiency related to two synthetic transcripts differing by the codon context around an amber codon. The codon contexts are derived from tobacco mosaic virus (TMV) and Moloney murine leukemia virus (Mo-MuLV) RNAs. The Mo-MuLV-like codon context does not promote suppression. Substituting TMV-derived triplets in the Mo-MuLV-like codon context shows that the two codons downstream from the TMV UAG signal are important determinants of suppression, as recently demonstrated in vivo.

Viral gene expression; RNA virus; Regulation of translation; Nonsense suppression; Codon context; Amber codon

1. INTRODUCTION

An increasing number of '+' strand RNA viruses (animal viruses such as retroviruses and alphaviruses, and plant viruses such as luteoviruses, carmoviruses, tymoviruses, tobamoviruses and tobraviruses) are known to regulate synthesis of their proteins via translational suppression of a termination codon [1–4].

Tobacco mosaic virus (TMV) synthesizes a 183K fusion protein that harbours the RNA-dependent RNA polymerase consensus motif [5], via suppression of the amber codon located at the end of the nonstructural 126K protein gene [6,7]. Moloney murine leukemia virus (Mo-MuLV) expresses its *pol* gene in the form of a gag-pol precursor protein upon readthrough by a $tRNA^{Gln}$ of the UAG codon terminating the *gag* cistron [8–10].

Abbreviations: CAT, chloramphenicol acetyltransferase; Mo-MuLV, Moloney murine leukemia virus; nt, nucleotide; ORF, open reading frame; TMV, tobacco mosaic virus; TYMV, turnip yellow mosaic virus; RF, release factor.

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Although the nature of the amino acid inserted into the TMV 183K protein during the in vivo readthrough process is not yet known, several isoacceptor tRNAs ($tRNA^{Tyr}$, $tRNA^{Gln}$, $tRNA^{Leu}$) from different sources have been shown to stimulate synthesis of this protein in vitro [11–17 and reviewed in 1, 4 and 18]. We have observed [2] that unrelated plant RNA viruses share homologous sequences flanking the suppressed UAG codons. Particularly striking is the homology found between these regions in luteovirus and carmovirus RNAs, and the identity of the sequence, CAA.UAG.CAA.U, found in TMV, turnip yellow mosaic virus (TYMV) and beet necrotic yellow vein virus RNAs.

These observations have prompted us to investigate the effect of codon context, for long recognized in prokaryotes (reviewed in [19]), on the efficiency of viral amber suppression. We have designed an in vitro approach using TMV and Mo-MuLV as a model system. We have constructed two synthetic genes carrying a short open reading frame (ORF) interrupted by an amber codon and located upstream of a reporter gene. These two constructs differ by the UAG context (two codons upstream and two codons downstream from the suppressed codon). The corresponding in vitro transcripts were used to program a rabbit reticulocyte translation system, with or without amber suppressor tRNA, and readthrough levels were analyzed. Constructs with chimeric codon contexts, comprising TMV- and Mo-MuLV-borne triplets, were also analyzed in view of identifying the determinants of TMV RNA suppression.

2. MATERIALS AND METHODS

2.1. *Oligonucleotides*

These were synthesized on a Biosearch Model 8600 DNA synthesizer and purified by 7 M urea–20% PAGE, pH 8.3. They were visualized by UV shadowing, excised from the gel and eluted overnight at 42°C in 0.4 M NH_4OAc. After desalting on NAP-25 columns (Pharmacia), the concentration was determined at A_{260} and the material lyophilized. Plasmids and transcripts are referred to with the number of the relevant oligonucleotide that discriminates them, preceded by the letter 'p' or 't' respectively.

2.2. *Cloning of mini-gene-CAT fusion plasmids*

Each oligonucleotide (600 pmol) was phosphorylated at its 5' end using T4 polynucleotide kinase (NEN) in a 30 μl reaction mixture. Oligonucleotide assembly (Fig. 1) was performed by combining 3 μl (1.5 μM) of each of four appropriate overlapping phosphorylated oligonucleotides in 40 μl of H_2O, heating the mixture at 80°C for 20 min and cooling to room temperature. The partially duplex structure was extended into a fully double-stranded product via fill-in (T7 DNA polymerase, Pharmacia) and ligation (T4 DNA ligase, Boehringer Mannheim) steps.

First, the two basic mini-genes 203 and 204 were constructed, featuring an ORF interrupted by an amber codon located in the TMV and Mo-MuLV codon context respectively (Fig. 1). They were cloned into the *Hin*dIII and *Bam*H1-digested pT3-T7α19 vector (BRL). An *Xba*I fragment containing the chloramphenicol acetyltransferase (CAT) reporter gene (gift from C. Robaglia, I.N.R.A., Versailles) was inserted into the unique *Xba*I site of the recombinant vector creating plasmids p203 and p204, in which the CAT ORF is located downstream of, and in-frame with the amber codon of the mini-gene.

Eleven additional mini-genes (Table I) were then constructed essentially as described above. Using oligonucleotides 205, 202 and 206 as common elements, and replacing oligonucleotides 203 or 204 by oligonucleotides harbouring different contexts around the amber codon, mini-genes 784, 785 and 788 to 792 were generated. In mini-genes 781 and 782, the amber codon contained respectively in oligonucleotides 203 and 204 was replaced by a Ser codon. In mini-genes 783/10 and 783/20, the amber codon contained in the TMV context was replaced by the ochre and opal codon respectively. Mini-genes 781 to 792 were restricted with *Hin*dIII and *Sal*I and ligated into similarly digested p204, creating p781 to p792.

Plasmids p203* and p204* were obtained by site-directed mutagenesis (Bio-rad kit) on the parental plasmids with an oligonucleotide that transforms the initiator Met codon of the CAT ORF into a Ser codon (TCT).

Cloning was achieved using standard techniques. The sequence of all constructs was verified by dideoxynucleotide sequencing (T7 sequencing kit, Pharmacia).

2.3. *In vitro transcription and translation*

Plasmids were prepared and purified using pZ523 columns (5 Prime –> 3 Prime, Inc.) as indicated by the supplier, linearized at the *Sma*I site located downstream of the CAT ORF and transcribed with T7 RNA polymerase (BRL). Transcription of the DNA (1–2 μg) was performed in 50 μl containing 50 μM (final concentration) GTP, 500 μM of each of the other NTP, 500 μM m^7GpppG (Boehringer Mannheim), 100 μg/ml of bovine serum albumin (BRL), 1–2 U of RNasin (Promega Biotec) and 250 U of T7 RNA polymerase (BRL). After incubation for 1 h at 37°C, 100 U of polymerase were added and incubation persued for 30 min at 37°C. The template DNA was then removed by incubation for 15 min at 37°C with 1–2 U of RQ_1 DNase (Promega Biotec). RNA was purified by passage through Sephadex G-50 columns followed by phenol and chloroform extractions, and ethanol precipitation. The amount, size and integrity of the RNA recovered was estimated by native agarose gel electrophoresis. The structure of the basic t203 and t204 is shown in Fig. 2.

Capped transcripts (0.2 μg) were translated for 1 h at 30°C using 550 KBq of [^{35}S]methionine (>30 TBq/mmol, Amersham) in 10 μl containing 5 μl of home-made rabbit reticulocyte lysate, as previously described [17]. Unfractionated yeast tRNA [23] from an amber suppressor strain that contains a Ser-inserting isoacceptor species (referred to as yeast amber suppressor tRNA) was provided by N. Wills (Howard Hughes Medical Institute, Salt Lake City, USA) and M. Tuite (University of Canterbury, UK); it was added to the in vitro translation mixture at 100 μg/ml (final concentration) where indicated. Two μl of the translation products were precipitated by 5% hot trichloroacetic acid to determine the total amount of radioactivity incorporated; the remaining 8 μl were analyzed by 0.1% SDS–12.5% PAGE. The dried gels were exposed using RX films (Fuji) for about 20 h. Quantification of suppression efficiency was achieved by cutting the relevant bands from the dried gel and counting the radioactivity they contain in 2 ml of scintillation fluid (Econofluor, NEN).

The control transcript tcat was derived from a p203 variant (in which the *Xba*I fragment bearing the CAT gene was inserted in the opposite orientation) by linearization with *Hin*dIII and transcription with T3 RNA polymerase (BRL). In tcat, the CAT ORF is flanked at its 5' and 3' end by 98 and 176 untranslated nucleotides respectively. No initiator AUG codon other than that of the CAT ORF being present, tcat translation is expected to yield uniquely the CAT protein.

3. RESULTS AND DISCUSSION

When translated in vitro (Fig. 3), t203 and t204 direct the synthesis of a major translation product (lanes 1 and 9) identified as the CAT protein of 25.6K [24], since it comigrates with the translation product of the tcat con-

Table I

Transcripts and efficiency of suppression[1]

Transcripts	Codon context	Suppression (%)
t204	gau.gac.**UAG**.gga.ggu	1.8
t785	gau.CAA.**UAG**.gga.ggu	2.3
t784	gau.gac.**UAG**.CAA.ggu	2.8
t788	gau.CAA.**UAG**.CAA.ggu	3.7
t789	ACA.CAA.**UAG**.gga.ggu	5.2
t791	ACA.CAA.**UAG**.CAA.ggu	7.3
t790	gau.gac.**UAG**.CAA.UUA	19.6
t792	gau.CAA.**UAG**.CAA.UUA	32.8
t203	ACA.CAA.**UAG**.CAA.UUA	31.4
t783/10	ACA.CAA.**UAA**.CAA.UUA	10.6
t783/20	ACA.CAA.**UGA**.CAA.UUA	69.2
t781	ACA.CAA.**UCA**.CAA.UUA	100
t782	gau.gac.**UCG**.gga.ggu	134

[1] Transcripts t203 and t204 contain the TMV-like and the Mo-MuLV-like codon contexts respectively. Codon context is defined as comprising the two upstream and two downstream codons flanking the internal UAG triplet. Transcripts t784, t785, and t788 to t792 harbour various triplet substitutions around the amber codon with respect to constructs t203 and t204. Transcripts t783/10 and t783/20 contain an ochre and an opal codon respectively in the TMV-like context in place of the amber codon. In transcripts t781 and t782, a Ser (UCG) codon replaces the amber codon present in t203 and t204. Percentage of suppression was calculated from the ratio of cpm contained in the suppression product synthesized in the absence of suppressor tRNA to the total hot trichloroacetic acid precipitable cpm deposited onto the gel (7.6×10^4–1.5×10^6 cpm; a blank value of 5400 cpm was deduced in all cases), the value obtained with transcript t781 being taken as 100% suppression. Codons from the TMV-like context are in upper- case letters, those from the Mo-MuLV-like context in lower-case letters, and internal triplets in bold upper-case letters.

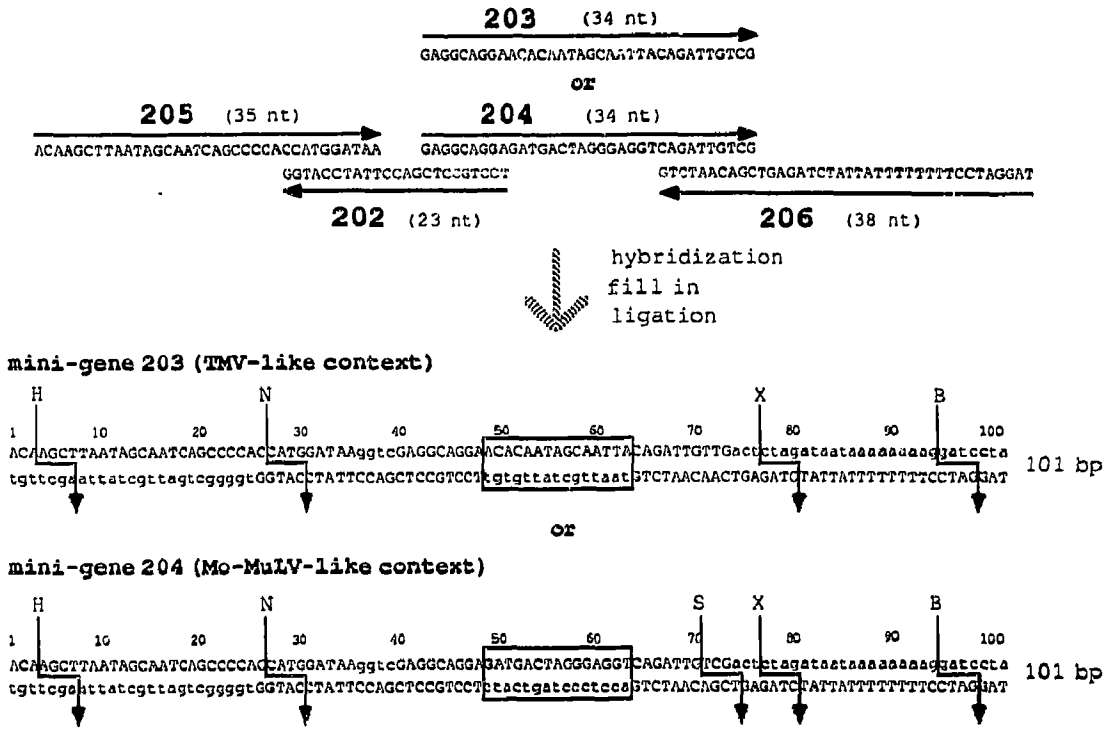

Fig. 1. Schematic diagram of the assembly of mini-genes 203 and 204. Oligonucleotides 205, 203, 202 and 206, and oligonucleotides 205, 204, 202 and 206 (the sizes of which are indicated in parentheses) with overlaps of 10 or 11 bp, were hybridized and extended by fill-in and ligation, yielding mini-genes 203 and 204, respectively. The sequence of the mini-genes is indicated (the sequence resulting from the fill-in reaction is in lower case letters). Mini-genes 203 and 204 differ by the codon context surrounding the internal amber codon: the TMV-like and the Mo-MuLV-like codon contexts are boxed. Using this strategy, mini-genes with modified codon contexts were obtained by replacing oligonucleotides 203 or 204 in the assembly reaction by oligonucleotide variants (Table I). Restriction sites are as follows: H=*Hin*dIII; N=*Nco*I; S=*Sal*I; X=*Xba*I and B=*Bam*HI.

trol (lane 7). In addition, synthesis of this product is abolished when translation is programmed by t203* (lane 5) or t204* (lane 13) in which the initiator Met codon of the CAT cistron was changed to a Ser codon. Translation of t203 (lane 1), but not of t204 (lane 9) yields an additional product (lower closed arrowhead) migrating more slowly than the CAT protein.

Translations were also performed in the presence of unfractionated yeast amber suppressor tRNA. Addition of such tRNA does not modify the translation pattern of t203 (comapre lanes 1 and 2). On the contrary when the suppressor tRNA is included in a reaction containing t204 as template (lane 10), a new minor product is present in addition to the CAT protein (upper closed arrowhead).

These more slowly migrating proteins have been identified as the expected suppression proteins on the basis of the following criteria. (i) Their size is larger than that of CAT (the expected size off the suppression product is 29K). (ii) They comigrate with proteins produced by two control transcripts in which the amber codon in the TMV or in the Mo-MuLV codon context has been replaced by a Ser codon (Table I), namely t781 (compare lanes 1 and 2 to lanes 3 and 4) and t782 (compare lane 10 to lanes 11 and 12). (iii) They share common V8 protease cleavage peptides with CAT indicating that they contain the sequence of the latter (data not shown) (iv) They are not initiated at the AUG codon of the CAT reporter gene ORF, as evidenced by their presence upon translation of t203* (lanes 5 and 6) and t204* (lane 14). (v) The hypothesis of a C-terminal elongation of CAT is highly improbable since neither in the absence nor in the presence of amber suppressor tRNA does a translation mixture programmed by tcat lead to the synthesis of a more slowly migrating protein (lanes 7 and 8). Furthermore, inspection of the CAT gene sequence reveals several in- and out-of-frame termination codons (TAA and TGA) following the TAA codon terminating the CAT cistron [20]. As discussed below, the difference in mobility observed in denaturing PAGE between the two suppression products (Fig. 3, upper and lower closed arrowheads) may be due to the difference in amino acid composition around the amber codon.

Apart from the unexpected synthesis of the CAT protein (not further discussed here) from an internal initiation codon (the AUG codon of the CAT cistron), a major conclusion can be drawn from these experiments. Efficient amber suppression is promoted in this system by the TMV-like context, since t203 leads to the synthesis of the suppression product even in the absence of added suppressor tRNA. On the contrary, the Mo-

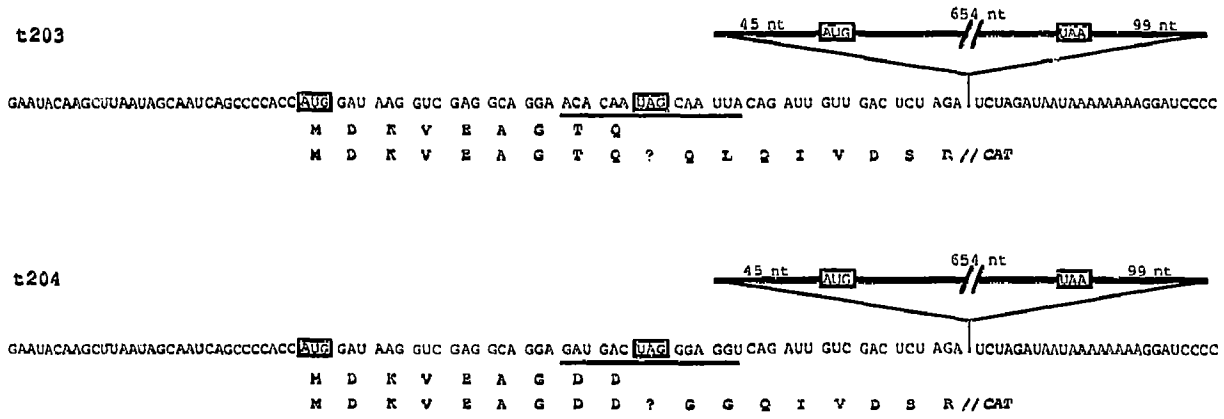

Fig. 2. Structure of transcripts t203 and t204. Mini-gene-CAT fusion plasmids p203 and p204 were linearized at the vector *Sma*I site and transcribed in vitro. The relevant nucleotide sequence of the corresponding transcripts t203 and t204 is shown. Of the CAT sequence [20] only the length of the coding region and of the 5'- and 3'-flanking regions is indicated. The transcripts are composed of a 31 nt-long untranslated sequence, a 753 nt-long ORF interrupted by an amber codon and terminated by the ochre codon of the CAT reporter gene, and a 125 nt-long 3'-untranslated sequence. The 5'-untranslated sequence comprises the 19 nt-long leader seqeunce of the TYMV coat protein mRNA [21] extended at its 5' end by 12 nt resulting from cloning and transcription procedures. Part of the coding region preceding the CAt cistron is derived from the original TMV RNA sequence [7] in both t203 and t204. Initiation and termination codons are boxed. The 5' proximal AUG initiation codon lies in the consensus sequence ACCAUGG favourable for initiation of translation [22]; the CAT initiator codon is conserved in these constructs. Codon contexts around the suppressible amber codon are underlined. In the absence of suppression, translation of the 753 nt-long transcript is expected to yield a 9 amino acid-long peptide and, if suppression occurs, an additional 251 amino acid-long product derived from the mini-gene-encoded sequence fused to the CAT ORF. Amino acids are indicated in the one-letter code.

MuLV-like codon context (defined here as composed of the two codons upstream and the two codons downstream of the amber codon) is not sufficient to promote readthrough in the in vitro conditions used here. Indeed, it has recently been shown that the 57 nt downstream of the UAG codon involving a pseudoknot structure, are required for suppression of the Mo-MuLV UAG codon to occur [25,26].

Ochre and opal codons embedded in the TMV-like codon context in place of the amber codon as in t783/10 and t783/20 can also be suppressed (Table I and Fig. 4, lanes 3 and 4). Endogenous opal suppression scores as the most efficient (69.2%), ochre and amber suppression reaching 10.6% and 31.4%, respectively.

Termination codon replacement experiments show that a full-length TMV clone remains fully infectious and leads to detectable levels of readthrough protein in vivo when the natural amber codon is changed to an ochre codon [27]. Ochre and opal codons are also leaky in tobacco protoplasts (to about the same extent although to a lesser extent than the amber codon) when present in the TMV-like context abutted to the β-glucoronidase reporter gene [28]. Similarly, replacement of the original termination codon with any of the other termination codons does not affect infectivity of Sindbis virus RNA [29] or Mo-MuLV RNA [30]. In the latter case, ochre and opal suppression was also observed in a rabbit reticulocyte lysate supplemented with tRNAs using transcripts extending far beyond the *gag* termination codon [30,31].

The availability of a suppressible versus a nonsuppressible codon context has provided us with a tool to determine the contribution of each codon in the

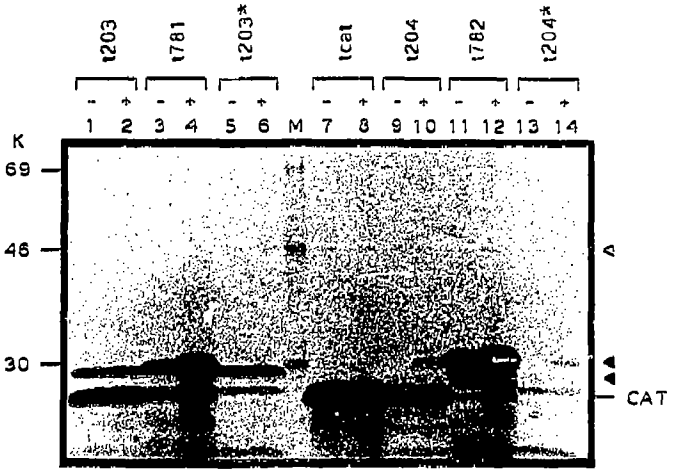

Fig. 3. Translation in vitro of transcripts carrying the TMV-like and Mo-MuLV-like codon contexts around the internal UAG codon. t203 (TMV-like), t204 (Mo-MuLV-like) and their corresponding control transcripts t203* and t204* (in which the initiator codon of the CAT ORF was mutated to a Ser UCU codon), tcat (template for the synthesis of CAT, used as size marker), and t781 and t782 (in which a Ser UCG codon replaces the amber codon) were translated in a reticulocyte lysate without (−) or with (+) yeast amber suppressor tRNA, and the translation products analyzed by SDS-PAGE. Transcripts are as designated above the lanes. To the left are given the size and position of the ^{14}C-labelled protein markers (M; Amersham), and to the right the position of CAT. Closed arrowheads point to the predicted suppression products, and the open arrowhead to an endogenous protein of the lysate that becomes labelled.

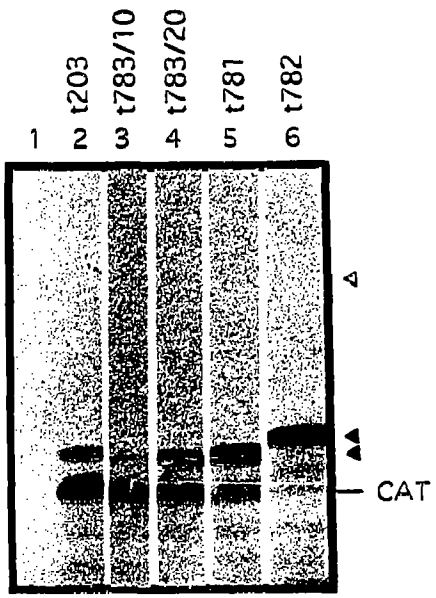

Fig. 4. Suppression in vitro of ochre and opal codons in the TMV-like context. Transcripts t783/10, t783/20 and t781 are t203 derivatives in which the amber codon was replaced by an ohcre, opal or serine codon respectively (Table I); they were translated in a reticulocyte lysate, and the products analyzed by SDS-PAGE. A sample with transcript t782, a derivative of t204 (Mo-MuLV-like context) has been included as reference for suppression product migration. Transcripts are as designated above the lanes. All wells are from the same gel. Lane 1: no RNA. Other indications are as in the legend of Fig. 3.

readthrough phenomenon, by analyzing the effect of successive triplet substitutions in the Mo-MuLV context (Table I and Fig. 5). These experiments were performed without suppressor tRNA to determine suppression efficiency, and with suppressor tRNA to better identify the suppression product. Low levels of readthrough observed for some transcripts in the absence of suppressor tRNA (t204, t785, t784, t788, t789 and t791; see Fig. 5 and below) could actually be quantified as described in Table I. For these transcripts, a 1-to 4-fold increase in suppression was reached by the addition of suppressor tRNA.

Replacing in t204 the codon 5' from the amber codon (t785), the codon 3' from the amber codon (t784) or both codons (t788) with the TMV-like CAA codon, only marginally increases suppression (Fig. 5, compare lanes 3, 5 and 7 to lane 1). Likewise, replacing the upstream context of t204 by the TMV-encoded ACA.CAA sequence (t789) only very weakly enhances suppression (lane 9). On the contrary, efficient readthrough is observed when the downstream Mo-MuLV context is changed to the TMV counterpart, CAA.UUA (t790, lane 11), indicating the suppression is dependent at least on these two codons.

Readthrough (lane 13 and Table I) remains low when the CAA codon 3' from the UAG triplet is present together with the upstream TMV context as in t791, leaving only one codon of Mo-MuLV origin at the very 3' end of the context. However, suppression tends to reach wild-type TMV levels in the opposite combination as in t792, in which the upstream CAA codon is present together with the downstream TMV context (lane 15).

The present results suggest that the major determinants of TMV suppression lie within the codons located 3' from the suppressible codon. Whereas replacing in t204 the first triplet 3' from the amber codon by the corresponding TMV triplet (t784) has little effect on the level of suppression, replacing both triplets 3' from the amber codon (t790) leads to a significant increase in

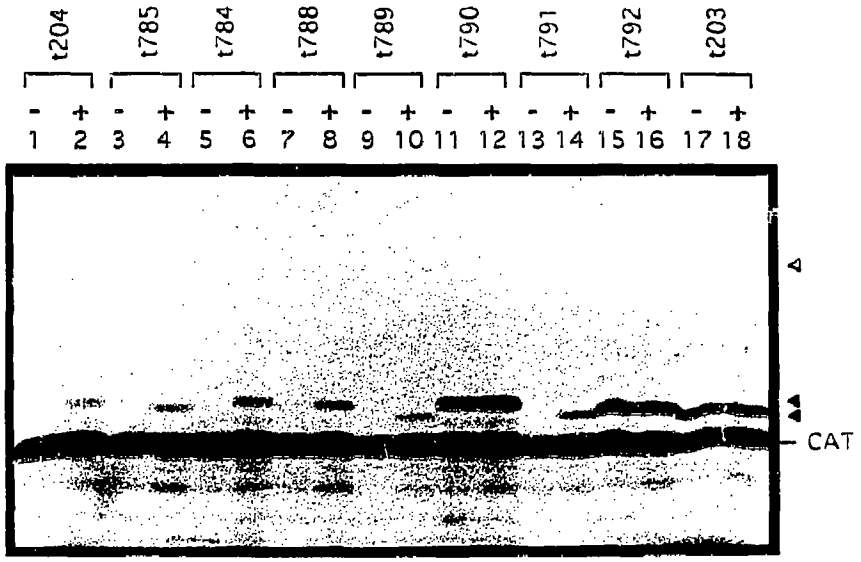

Fig. 5. Effect on UAG suppression of TMV and Mo-MuLV codon swapping. Transcripts harbouring TMV or Mo-MuLV chimeric codon contexts around the amber codon (Table I) were translated in vitro without (−) or with (+) suppressor tRNA, and the products analyzed by SDS-PAGE. Transcripts are as designated above the lanes. Other indications are as in the legend of Fig. 3.

suppression, thereby stressing the importance of at least the second triplet following the UAG codon for efficient readthrough. Skuzeski et al. [28] have recently demonstrated that in vivo the leaky TMV context resides in the sequence UAG-CAR-YYA. Our in vitro results confirm the requirement for the codons downstream of the TMV RNA amber triplet for efficient suppression, and also indicate that the upstream codons may contribute to optimize suppression efficiency. A full mutagenesis panel is required to confirm this observation.

Similar results to those presented above on the determinants of suppression were obtained using wheat germ extracts containing RNasin and no exogenous tRNA other than the suppressor tRNA. The appearance of the readthrough product upon translation of t203 in a wheat germ system in the absence of added suppressor tRNA, is consistent with results obtained when TMV genomic RNA serves as template in the above translation conditions (unpublished results). Readthrough is however, more efficient with the transcript than with the viral RNA.

As mentioned above and as discussed here (and data not shown), the difference in electrophoretic mobility between the t203 and t204 readthrough proteins, seems to depend on the vicinity of the two negatively charged Asp residues (codons GAU.GAC). The suppression product synthesized by transcripts containing the two Asp residues (t204, t784, t790 and t782) migrates as a heavier band, the one produced by transcripts containing Thr.Gln residues (codons ACA.CAA, as in t203, t789, t791, t783/10, t783/20 and t781) migrates as a lighter band, and the one produced by transcripts containing one Asp residue only in the form Asp.Gln (codons GAU.CAA as in t785, t788 and t792) migrates in an intermediate position.

4. CONCLUSIONS

The in vitro system presented here can be used to study the determinants of nonsense suppression for a variety of viral RNA candidates. It is also conceivable to use it in future studies of release factor (RF) function and recognition.

The fact that all termination codons can be suppressed in a given viral context suggests an intrinsic leakiness of those sequences. This may explain why several different natural suppressor tRNAs were shown to read through the amber codon of TMV RNA in vitro. As pointed out earlier [2], striking similarities in the sequences around the amber codon are observed among plant viral RNAs undergoing natural suppression of termination codons.

This observation adds to the concept of a codon context effect in translational readthrough of termination codons. Such a context may assume different forms: a linear hexameric downstream determinant as in TMV RNA ([28] and this paper), a downstream 40 nt long sequence comprising a stem and loop structure as in the case of the insertion of Se-Cys in the *Escherichia coli* formate dehydrogenase mRNA [32], or a downstream signal composed of a linear eight nucleotide sequence followed by a pseudoknot structure as in Mo-MuLV RNA suppression [25,26]. Whether the molecular basis of this effect involves tRNA-tRNA [33], rRNA-mRNA [34], mRNA-RF [2,35], or more complex competitive interactions such as suppressor tRNA-ribosomes-mRNA versus RF-ribosomes-mRNA [2] is not yet known. Further studies are needed to reveal the interplay between the components of the translational apparatus in the readthrough phenomenon.

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