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Attenuation of Theiler's Murine Encephalomyelitis Virus by Modifications of the Oligopyrimidine/AUG Tandem, a Host-Dependent Translational *cis* Element

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A set of Theiler's murine encephalomyelitis virus mutants with engineered alterations in the conserved oligopyrimidine/AUG tandem (E. V. Pilipenko, A. P. Gmyl, S. V. Maslova, G. A. Belov, A. N. Sinyakov, M. Huang, T. D. K. Brown, and V. I. Agol, J. Mol. Biol. 241:398–414, 1994) were assayed for their growth potential in BHK-21 cells (as reflected in plaque size) and for neurovirulence upon intracerebral inoculation of mice. Tandem-destroying mutations, which included substitutions in the oligopyrimidine moiety and extended insertions into the oligopyrimidine/AUG spacer, exerted relatively little effect on the plaque size but ensured a high level of attenuation. The attenuated mutants exhibited remarkable genetic stability upon growth in BHK-21 cells. However, the brains of rare animals that developed symptoms after the inoculation with high doses of these mutants invariably contained pseudorevertants with the oligopyrimidine/AUG tandem restored by diverse deletions or an AUG-generating point mutation. The AUG moiety of the tandem in the revertant genomes was represented by either a cryptic codon or initiator codon. The results demonstrate that the tandem, while dispensable for the Theiler's murine encephalomyelitis virus growth in BHK-21 cells, is essential for neurovirulence in mice. Thus, the oligopyrimidine/AUG tandem is a host-dependent *cis*-acting control element that may be essential for virus replication under certain conditions. The functional activity of the tandem was retained when its oligopyrimidine or AUG moieties were made double stranded. A possible role of the tandem in the cap-independent internal initiation of translation on the picornavirus RNA templates is discussed.

Picornaviruses, small naked RNA-containing animal viruses, exploit cap-independent translation initiation for the synthesis of their proteins. Several genomic *cis*-acting elements are required for this process. Two of these elements, namely, the internal ribosome entry site (IRES) and the starting window, are indispensable components of the genomes of all viruses belonging to this family. IRES ensures, with the aid of a variety of protein factors, initial binding of a ribosome (or a ribosomal subunit) to the template; the element resides in a several-hundred-nucleotide (nt)-long nonterminal segment of the 5′-untranslated region (5UTR) of viral RNA (1, 21, 26). The starting window is a recently discovered element corresponding to a locus with which an IRES-bound ribosome makes productive contacts allowing its 5′→3′ movement along the template (translation or scanning, depending on the presence or absence of an AUG codon in a favorable context) (17); in the case of Theiler's murine encephalomyelitis virus (TMEV) RNA, the starting window is a dozen nt in length and is located ~16 to 17 nt downstream of IRES (17). A third *cis* element, an appropriately spaced oligopyrimidine/AUG tandem, known to be conserved among picornaviruses (3), is essential for efficient translation and reproduction of poliovirus (4, 6, 12–14, 18). An unambiguous proof of the latter notion was obtained with poliovirus mutants having engineered insertions or deletions between the two moieties of the tandem, the oligopyrimidine (box A) and AUG (cryptic AUG-586 lying within a specific context, box B, ~150 nt upstream of the initiator AUG-743) (4, 18). These mutations suppressed both the template activity of the mutant RNAs in cell-free translation systems and the growth potential of the virus, as evidenced by a significant decrease in the plaque size. The mutants, however, were genetically unstable, and upon in vitro cultivation, they readily produced large-plaque-forming (pseudo)revertants with different genetic changes (point mutations, deletions, insertions), all of which tended to restore the appropriately spaced box A/AUG tandem with the AUG moiety represented by the authentic or a newly created cryptic AUG or the initiator AUG-743.

Surprisingly, a similar oligopyrimidine/AUG tandem has recently been demonstrated to be dispensable for some picornaviruses, judging by the efficient in vitro translation of TMEV and encephalomyocarditis virus RNAs with different engineered alterations in the tandem (9, 17) as well as by the viability and genetic stability of appropriate TMEV mutants in BHK-21 cells (17). TMEV and encephalomyocarditis virus, on the one hand, and poliovirus, on the other, belong to different genera of picornaviruses, namely, cardioviruses and enteroviruses, respectively. The two groups possess structurally unrelated IRESs, whereas the conservation of the IRES secondary structure within a given genus is very high (15, 16). Another difference between the cardioviruses and enteroviruses consists in distinct functions of the AUG moiety of the oligopyrimidine/AUG tandem. This AUG serves as the initiator codon for the polyprotein in the former case but is cryptic (noninitiator) in the latter one (while mapping to the starting window in both groups of viral genomes). However, this distinction does not appear crucial since, as already mentioned, the AUG moiety of the tandem may be represented by the initiator codon in a class of poliovirus mutants (4, 18).

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Although the above differences in the organization of the translational *cis* elements in cardioviruses and enteroviruses may well contribute to the apparent dissimilarity in the requirement for the tandem, the very fact of dispensability, in the case of cardioviruses, of a highly conserved genetic element was intriguing. Therefore, we decided to explore the possibility that the TMEV oligopyrimidine/AUG tandem, being redundant in some host systems, is essential in others; in particular, we wanted to know whether the tandem is needed for TMEV reproduction in its natural host, mice, and, more specifically, in the murine central nervous system. This question seemed to be warranted as examples of the host dependence of 5UTR mutations are well documented in different picornaviruses. One of the manifestations of such host dependence is the attenuation of neurovirulence, whereby the reproduction of a virus is specifically restricted in cells of the central nervous system. It was especially illuminating that some 5UTR mutations decreasing poliovirus neurovirulence impaired initiation of translation of poliovirus RNA (23–25), particularly in neural cells (11). Furthermore, poliovirus mutants lacking certain segments of the 5UTR exhibited no marked deficiency with respect to the growth in vitro, while being highly attenuated for susceptible animals (5, 20a). Similarly, determinants in the TMEV 5UTR involved in the control of translation initiation have been reported to affect neurovirulence, although no simple correlation between the template activity of the mutant viral RNA species in reticulocyte lysates and the level of neurovirulence of the corresponding viruses was observed (22). Certain modifications of the TMEV IRES markedly reduced neurovirulence, exerting only a relatively small effect on the growth characteristics of the mutant in tissue culture (2, 19).

Here, we report that alterations of any component of the tandem (the oligopyrimidine, AUG, or the distance between them) resulted in a dramatic decrease in the TMEV neurovirulence. In the mouse brain, as opposed to BHK-21 cells, the mutants with extended AUG-lacking insertions into the oligopyrimidine/AUG spacer proved to be genetically unstable, readily producing pseudorevertants with a restored arrangement of the tandem. These results demonstrate that the picornavirus requirement for the oligopyrimidine/AUG tandem is host dependent.

MATERIALS AND METHODS

Viruses and cells. BHK-21 cells were obtained from the Cell Culture Collection of the Ivanovsky Institute of Virology, Moscow, Russia. The construction of the TMEV mutants was described previously (17); the structures of the relevant segments of their genomes are given in Tables 1 and 2 and Fig. 3. Virus pools corresponded to the third or fourth blind passage after the initial transfection with the mutant transcripts.

Virus titration. Fifty percent tissue culture infective doses (TCD_{50}) were calculated with the Reed and Muench formula (19a) on the basis of microscopic observations of monolayer BHK-21 cultures infected with 10-fold dilutions of the virus, by using at least four cultures for each dilution; the results were scored on day 4.

Plaque phenotype. Two-day-old cultures of BHK-21 cells grown in 50-mm plastic dishes were infected and processed as described previously (17). On day 3, the cultures were stained with either crystal violet or neutral red. The sizes of 50 to 100 plaques were measured and averaged. The average size of plaques produced by wild-type GDVII, included in each assay as a standard, varied between 3.3 and 3.5 mm. For each mutant, the assay was performed at least twice.

One-step growth curve. One-day-old BHK-21 cell cultures grown as monolayers in vials for antibiotics ($\sim 2 \times 10^5$ cells per vial) were infected with respective viruses at an input multiplicity of 50 TCD_{50} per cell. After 1 h of adsorption at an ambient temperature, unadsorbed virus was washed off, 2 ml of the Eagle medium was added, and cultures were incubated at 37°C. At appropriate time intervals, the cultures were frozen and viral titers, after two additional cycles of freezing-thawing, were determined as described above.

Neurovirulence assay. BALB/c mice (7 to 8 g) were inoculated intracerebrally with 10-fold virus dilutions ($10^{1.5}$ to $10^{7.5}$ TCD_{50}), with eight animals for each

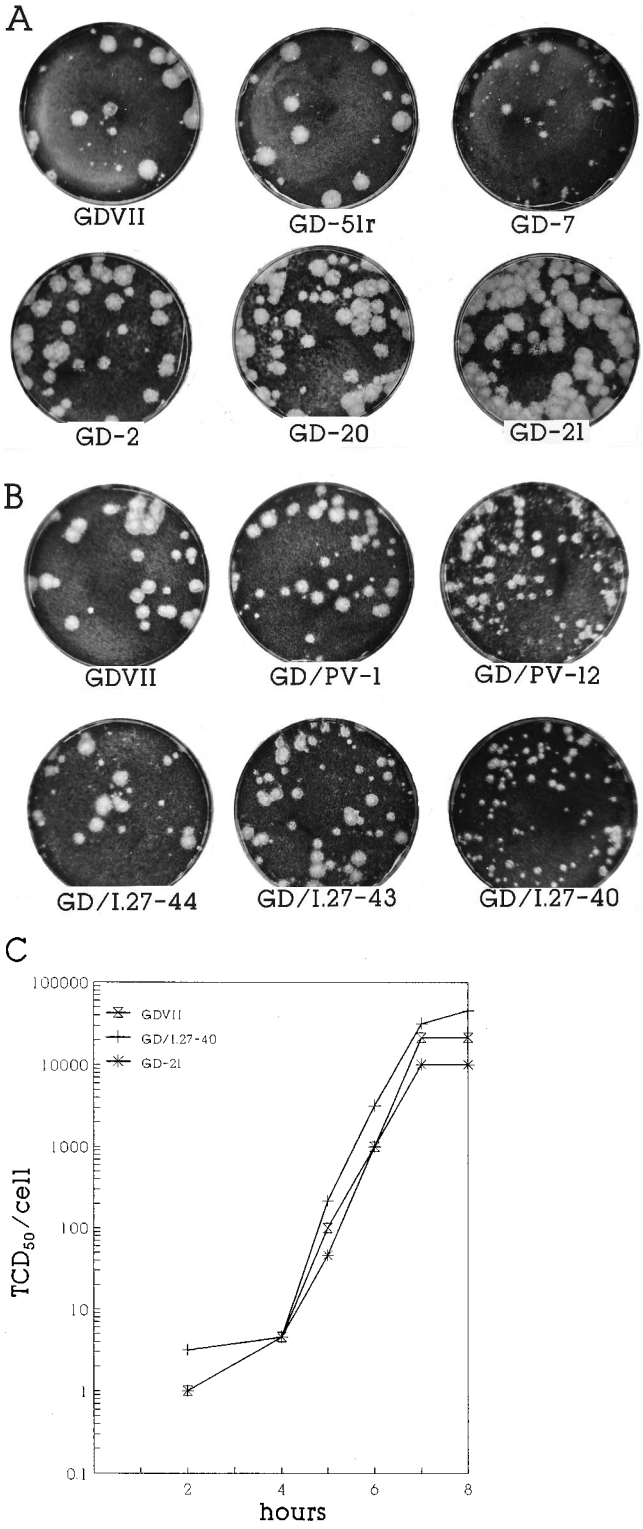

FIG. 1. Growth potential of TMEV mutants. (A and B) The plaque phenotypes of the mutants with substitutions (A) and insertions (B) in the oligopyrimidine/AUG tandem region. The infected cultures were stained with crystal violet on day 3. (C) One-step growth curves of the wild-type GDVII and mutants with a destroyed oligopyrimidine (GD-21) as well as with an AUG-lacking insertion (GD/I.27-40).

dilution. The animals were observed for clinical signs for 45 days. The paralytogenic activity was expressed as 50% paralytogenic doses (PD_{50}) calculated according to the Reed and Muench method (19a). The PD_{50} of GDVII, included in each experiment as a standard, varied between $10^{2.2}$ and $10^{2.7}$ TCD_{50}. Each mutant was assayed at least twice, and the difference between two determinations of PD_{50} did not exceed $10^{0.3}$ TCD_{50}.

RNA sequencing. The virus pools were checked by sequencing of the appropriate region (positions 910 to 1090) of the viral genome by the chain termination method and with avian myeloblastosis virus reverse transcriptase (16).

Analysis of the replicating virus. Brain samples were picked up from at least two diseased mice inoculated with a given mutant. The 10% brain suspensions were used to infect BHK-21 monolayers and/or for the PCR analysis of the relevant segment of the TMEV genome. Similarly, the material from neutral red-stained plaques generated by certain mutants was picked up and suspended in 1 ml of Earle's saline. PCR was carried out as described previously (4), with samples roughly equivalent to 1/1,000 part of the mouse brain or 1/100 part of the material from a plaque. Sense and antisense oligodeoxynucleotide primers corresponded to positions 779 to 798 and 1196 to 1215, respectively, of the wild-type GDVII RNA. Some 20 or 30 cycles of the amplification were performed for analytical and preparative purposes, respectively (only 15 cycles were done, if the starting material was represented by the purified viral RNA). The amplified DNA product to be sequenced was purified by agarose gel electrophoresis.

Electrophoresis under nondenaturing conditions. The amplified DNA segments were 5′ terminally labeled with [γ-^{32}P]ATP and phage T4 polynucleotide kinase followed by electrophoresis in 5% polyacrylamide gels (with an acrylamide-bisacrylamide ratio of 7:1).

Cloning of the PCR-amplified fragments. The virus-specific DNA fragments with coordinates 779 to 1215 derived from GD-40R3 and GD-40R9 were cloned into pBSM13$^-$ (an M13 phage-based transcription vector; Vector Cloning Systems, San Diego, Calif.) by ligation with blunt ends generated after the *Hin*dIII digestion and filling in with the Klenow enzyme.

DNA sequencing. Sequenase version 2.0 (U.S. Biochemical, Cleveland, Ohio) was used according to the protocol recommended by the manufacturer. In the case of PCR-amplified DNA, the reaction mixture contained 10% dimethyl sulfoxide.

RESULTS

Plaque phenotype of mutants with altered oligopyrimidine/AUG tandem. In the TMEV RNA, the oligopyrimidine/AUG tandem lies just downstream of IRES and partially overlaps the starting window (17); its AUG moiety corresponds to the initiator codon of the polyprotein reading frame. The engineered modifications of the tandem (nucleotide substitutions in the oligopyrimidine or extended insertions into the oligopyrimidine/AUG spacer) brought about only slight, if any, inhibition of in vitro translation of the altered GDVII RNA species; the mutant viruses were not only viable but proved to be genetically stable in BHK-21 cells (17).

Substitutions in the 3′-proximal (GD-2 and GD-19) or 5′-proximal (GD-20) parts of the oligopyrimidine, in both of these elements (GD-21), or in the oligopyrimidine/AUG spacer (GD-9) resulted in only a small (<25%), if any, decrease in the plaque size generated on BHK-21 cells. A somewhat greater, but still not dramatic, suppression of the growth potential was observed for a mutant with four pyrimidines deleted (GD/Δ4-54) (Fig. 1A; Table 1). The destruction of the oligopyrimidine did not appreciably affect the one-step growth curve either (Fig. 1C). The relevant portion (nt 779 to 1196) of the PCR-amplified material from three large plaques generated by GD-20 and GD-21 was sequenced, and no alterations compared with the expected mutant sequences were found (not shown).

Similarly, destruction of the tandem by insertions, into the oligopyrimidine/AUG spacer, of AUG-lacking 27-nt-long (GD/I.27-40) or 108-nt-long (GD/PV-12 and -22) RNA segments was reasonably well tolerated, as judged by the plaque phenotype of the relevant mutants: the smallest average plaque size (approximately twofold reduction relative to GDVII) was observed in GD/I.27-40 (Fig. 1B; Table 2). Introduction, into the inserts, of AUG in a good (GD.I/27-44) or poor (GD.I/27-43 and GD/PV-1 and -11) context somewhat increased the size of plaques regardless of the context (Fig. 1; Table 2). No adverse effect of an AUG-lacking insertion on the one-step growth curve could be registered (Fig. 1C).

Thus, the plaque phenotype of the mutants gave additional, more quantitative support for the notion (17) that the oligopyrimidine/AUG tandem is not essential for TMEV reproduction in BHK-21 cells.

Neurovirulence of the mutants with altered oligopyrimidine/AUG tandem. As indicated in the Introduction, we hypothesized that, despite the dispensability of the TMEV oligopyrimidine/AUG tandem in several in vitro systems, this element might be important for the reproduction of the virus in its natural host. Therefore, the relevant mutants were assayed for neurovirulence upon intracerebral inoculation of mice. Substitutions in the 3′-proximal portion of the oligopyrimidine (GD-2 and -19) or in the oligopyrimidine/AUG spacer (GD-9) did not significantly affect the PD_{50}, in accord with the lack of a marked effect of these mutations on the plaque phenotype (Table 1). However, some other alterations in the oligopyrimidine, e.g., a 4-nt deletion in its 3′-proximal part (GD/Δ4-54) or a substitution in the 5′-proximal part (GD-20), brought about a moderate attenuating effect manifested as an increase in the PD_{50} 10 and 100 times, respectively. The identity of the inoculated mutants with the viruses replicating in the brains of the diseased mice was confirmed by sequencing of the PCR-amplified material from at least two animals for each mutant. The relevant segment of the genomes underwent no changes despite the fact that the brain samples were taken in some cases as late as 29 to 33 days after the inoculation with a small dose of the mutant (not shown).

TABLE 1. GDVII viruses with mutations in the IRES-AUG spacer and their biological properties

Name	Sequence[a]	Box A-AUG distance	Plaque size (mm)	PD_{50} (log_{10})
	1042 ↓ 1071 ↓			
GDVII	UUUUCUCUUUUUAUUAUAUUGUCAAUAUGG	21	3.3	2.5
GD-2	UUUUCggaUccUAUUAUAUUGUCAAUAUGG	21	2.8	2.3
GD-19	UUUUCcgcagUUAUUAUAUUGUCAAUAUGG	21	3.1	2.0
GD-20	UUagaUCUUUUUAUUAUAUUGUCAAUAUGG	16[b]	3.0	4.3
GD-21	UUagacgcagUUAUUAUAUUGUCAAUAUGG	NR[c]	3.1	>7.5
GD/Δ4-54	UUUUCg****cUAUUAUAUUGUCAAUAUGG	17	2.1	3.5
GD-9	UUUUCUCUUUUguaauauauauUCAAUAUGG	21	2.6	2.8

[a] The mutated and deleted nucleotides are printed in lowercase and marked by asterisks, respectively. The upstream portion of the oligopyrimidine stretch (box A) and the authentic start codon are underlined with thick lines; the oligopyrimidine downstream portion (box A′) is underlined with a thin line.

[b] The box A′-AUG distance.

[c] NR, not relevant.

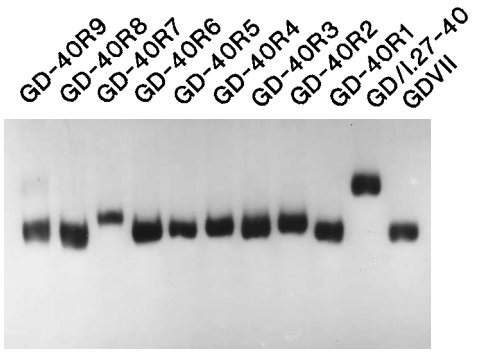

FIG. 2. Nondenaturing polyacrylamide gel electrophoresis of PCR-amplified fragments (corresponding to positions 779 to 1215 of the wild-type genome) of the mutant TMEV RNAs. RNA preparations used as templates for PCR were isolated from virus pools in the case of GDVII and GD/I.27-40 (the third and ninth passages in BHK-21 cells, respectively) and from brain suspensions of diseased animals in the case of GD-40R1 through GD-40R9. The amplified DNA was terminally labeled and analyzed as described in Materials and Methods.

TABLE 2. GDVII viruses with extended insertions and their biological properties

Name	Sequence[a]	Box A-AUG distance	Plaque size (mm)	PD_{50} (log_{10})
	1040 1046 1053 1073			
GDVII	GGUUUUC UCUUUU.....UAUUAUAUUGUCAAUAUGGCU	21	3.3	2.5
GD/I.27-40	GGUUUUC GGAUCUUUUAUUUUAUUGAAUUAGAUCUGAUCC.....UAUUAUAUUGUCAAUAUGGCU	48	1.7	7.7
GD/I.27-43	GGUUUUC GGAUCUUUUAUUUUAUUGuAUauGuUCUGAUCC.....UAUUAUAUUGUCAAUAUGGCU	21	2.6	5.2
GD/I.27-44	GGUUUUC GGAUCUUUUAUUUUAUUCAAUauGgcuaGAUCC.....UAUUAUAUUGUCAAUAUGGCU	21	2.6	3.7
	569 675			
GD/PV-1	GGUUUUC GGAUCUGUUUAUUGUGGCUGCUUAUGGUGACAAUCACAGAUUGUUAUCAUAA CC UAUUAUAUUGUCAAUAUGGCU	24	2.7	4.2
GD/PV-11	GGUUUUC GGAUCUGUUUAUUGUGGCUGCUUAUGGUGACAAUCACAGAUUGUUuagucuA CC UAUUAUAUUGUCAAUAUGGCU	24	2.5	4.5
GD/PV-12	GGUUUUC GGAUCUGUUUAUUGUGGCUGaaauUGGUGACAAUCACAGAUUGUUAUCAUAA CC UAUUAUAUUGUCAAUAUGGCU	129	2.3	>7.5
GD/PV-22	GGUUUUC GGAUCUGUUUAUUGUGGCUGCUUuUGGUGACAAUCACAGAUUGUUuagucuA CC UAUUAUAUUGUCAAUAUGGCU	129	1.9	>8.0

[a] The mutated nucleotides within the insertions are printed in lowercase. The AUGs within the insertions and the authentic start codon are underlined with thin and thick lines, respectively. Box A is underlined with a thick line. Dots are used for alignment purposes.

On the other hand, the mutant with an entirely destroyed oligopyrimidine (GD-21), being a large-plaque-former, proved to be highly attenuated, causing a slight transient paresis in a single mouse among the 30 animals inoculated with the highest dose of the virus ($10^{7.5}$ TCD_{50}) (Table 1). The appropriate segment of the viral genome in the brain homogenate of this mouse was subjected to PCR amplification followed by sequencing. The primary structure of the mutated region was fully preserved (not shown).

Extended AUG-lacking insertions into the oligopyrimidine/AUG spacer (GD/I.27-40 as well as GD/PV-12 and -22) resulted in a comparably severe loss of neurovirulence (Table 2). If, however, these insertions contained an AUG at a proper distance (21 to 24 nt) from the box A component (UUUUC) of the oligopyrimidine, the level of attenuation depended on the AUG context. PD_{50} of a mutant with a good-context AUG (GD/I.27.44) was only 10 times higher compared with that of GDVII (and even this difference could perhaps be attributed to lengthening of the leader polypeptide due to the fact that the engineered AUG was in frame with the viral polyprotein). An AUG context identical to that of poliovirus box B made the virus somewhat more attenuated regardless of the presence (GD/PV-11) or absence (GD/PV-1) of a terminator codon downstream of the engineered AUG. GD/I.27-43 having a poor-context AUG (non-Kozak, non-box B) exhibited an even greater level of attenuation, being, however, not as attenuated as the mutants with AUG-lacking insertions. The viruses with AUG-containing insertions retained the engineered alterations in vivo, as judged by sequencing of the PCR-amplified material from the brains of at least two diseased mice inoculated with each relevant mutant (not shown).

It should be emphasized that the actual level of neurovirulence of the mutants with the AUG-lacking extended insertions (GD/I.27-40 and GD/PV-12 and -22) should be significantly lower than suggested by the PD_{50} face values presented in Table 2; as shown below, the disease observed in the animals inoculated with these viruses appeared to be due to the emergence of virulent revertants.

Thus, the observations summarized in Tables 1 and 2 strongly suggested that it was the appropriately spaced oligopyrimidine/AUG tandem rather than its individual moieties that was crucial for the neurovirulence of GDVII. The functionally active tandem may contain either the 5′-proximal (box

TABLE 3. Genome structure of the revertants selected from GD/I.27-40

Name	Onset of paralysis		Sequence[a]	Box A-AUG distance
	Day	Dose[b]		
			1040 ↓ 1071 ↓	
GD/I.27-40			GGUUUUCGGAUCUUUUAUUUUAUUGAAUUAGAUCUGAUCCUAUUAUAUUGUCAAUAUGG	48
GD-40R1	11	7.8	GGUUUUCGGAUCUUUUA**********UAUAUUGUCAAUAUGG	22
GD-40R2	13	7.5	GGUUUUCGGAUCUUUUAUU**********UUAUAUUGUCAAUAUGG	25
GD-40R3	14	7.5	GGUUUUCGGAUCUUU**********UAUUAUAUUGUCAAUAUGG	23
GD-40R4	20	7.5	GGUUUUCGGAUCUUUUAUUU**********UAUUAUAUUGUCAAUAUGG	28
GD-40R5	21	6.5	GGUUUUCG**********GAUCUGAUCCUAUUAUAUUGUCAAUAUGG	26
GD-40R6	35	5.5	GGUUUUCGGAUCUUUU**********UUAUAUUGUCAAUAUGG	22
GD-40R7	18	7.8	GGUUUUCGGAUCUUUU**********AUCCUAUUAUAUUGUCAAUAUGG	28
GD-40R8	16	7.8	GGUUUUCGGAUCUUU**********UAUUAUAUUGUCAAUAUGG	23
GD-40R9	10	7.8	GGUUUUCGGAUCUUUUA**********UAUAUUGUCAAUAUGG GGUUUUCG**********GAUCUGAUCCUAUUAUAUUGUCAAUAUGG	22 26

[a] See Table 1, footnote *a*.
[b] The inoculation dose in $\log_{10}$ TCD_{50} that caused disease of the mouse from which the respective virus was isolated.

A) or 5′-distal (box A′) portions of the oligopyrimidine as well as either an initiator or a cryptic AUG.

Genome structure of the revertants isolated from mouse brains. The mutants with extended AUG-lacking insertions (GD/I.27-40 and GD/PV-12 and -22), being highly attenuated, nevertheless caused a paralytic disease in some of the mice inoculated with a large dose of the viruses. The PCR analysis performed with nine brain homogenates of individual GD/I.27-40-infected diseased mice (from different experiments) demonstrated, however, that the multiplying viruses differed from their parent. Compared with the inoculated mutant, all of them generated shorter DNA fragments with lengths more similar to that of the wild-type GDVII (Fig. 2). Sequencing of these PCR-amplified fragments demonstrated the presence of deletions, which invariably tended to restore the parental oligopyrimidine/AUG tandem arrangement by decreasing the box A-AUG distance from 48 nt in GD/I.27-40 to 22 to 28 nt in the revertants (Table 3) (compare with the wild-type GDVII value of 21 nt). In the case of revertant GD-40R9, a heterogeneity was revealed upon sequencing of the PCR-amplified genome segment. The segment was cloned into pBSM13^{-}. Deletions of 26 and 22 nt were observed for two and five clones, respectively (Table 3). A similar analysis of GD-40R3 demonstrated that eight clones were identical to each other, suggesting a high degree of homogeneity of the revertant.

Brain homogenates from five of the above nine mice (GD-40R1 through R5) were used to infect BHK-21 cells; in all cases, viable viruses were recovered. RNA sequencing of the relevant portion of the genomes of these viruses gave the results identical to those obtained with the PCR-amplified DNA fragments listed in Table 3 (not shown). Three of these isolates were assayed for neurovirulence and were found to lose their highly attenuated phenotype (Table 4). Notably, GD-40R1, the revertant with a more favorable box A-AUG distance of 22 nt, turned out to be more virulent than GD-40R2 and -40R4 (25 and 28 nt, respectively). In contrast to their parent (GD/I.27-40), the virulent revertants were genetically stable upon the reproduction in central nervous system, judging by the results of sequencing of (i) the PCR-amplified material from brain homogenates taken late after the intracerebral inoculation and (ii) the infectious viruses isolated from these brains (not shown).

As already shown, the engineered mutants with even longer AUG-lacking insertions, GD/PV-12 and -22, were also found to be highly attenuated (Table 2). Again, the PCR-cloning-sequencing analysis of brain homogenates of rare diseased animals revealed deletions decreasing the box A-AUG spacer from an unacceptable 129 nt to 19 to 26 nt (Table 5). In one case, GD/PV-22R1, the genetic change was represented by an AUG-generating point mutation rather than deletion; the spacer between box A and the newly created AUG in this revertant comprised 24 nt (Table 5).

A similar analysis failed to detect any deletions (or other alterations) in the relevant segment of the RNA from pools of GD/I.27-40 (Fig. 2), GD/PV-12, and GD/PV-22 (not shown) obtained after nine, four, and four passages, respectively, in BHK-21 cells.

Secondary structure of the oligopyrimidine/AUG tandem is not important. In a previously engineered set of GDVII constructs (17), two mutants had modified secondary structure in the region of the oligopyrimidine/AUG tandem (Fig. 3). The oligopyrimidine appeared to be essentially single-stranded (except for a few 5′-proximal bases) in the wild-type GDVII, but it was completely buried in a duplex in GD-7. This modification slightly depressed the plaque size and did not markedly affect the virulence (Fig. 3). When the initiator AUG in the tandem was made base paired (GD-51r), essentially no effect on either the plaque phenotype or virulence was registered (Fig. 3). Thus, there seems to be no strict requirement for a specific secondary structure (or its absence) of either component of the oligopyrimidine/AUG tandem.

DISCUSSION

The oligopyrimidine/AUG tandem is a host-dependent control element. The main finding of this work is the demonstration that the oligopyrimidine/AUG tandem, while dispensable for the TMEV growth in BHK-21 cells (and for translation of its RNA in Krebs-2 extracts and reticulocyte lysate [17]), is essential for neurovirulence. The complete destruction of the

TABLE 4. Biological properties of the GD/I.27-40 revertants

Name	Plaque size (mm)	PD_{50} ($\log_{10}$)
GDVII	3.3	2.5
GD-40R1	3.6	2.8
GD-40R2	3.4	4.2
GD-40R4	3.3	4.0

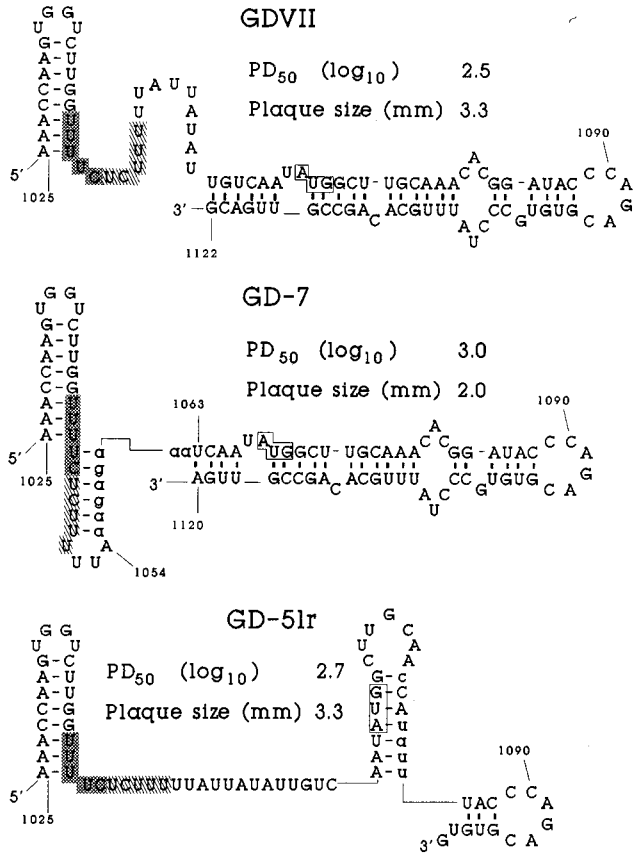

FIG. 3. Neurovirulence and plaque phenotype of TMEV mutants with altered secondary structures of the oligopyrimidine/AUG region. The secondary structure probing was carried out previously (17). GD-51r is a derivative of GD-51 which acquired a G-1078→A transition upon passages in BHK-21 cells (17). Box A and box A′ are cross-hatched and hatched, respectively; the initiator AUG is boxed.

oligopyrimidine, or the insertion of extended AUG-lacking spacers between the oligopyrimidine and the initiator AUG-1086, resulted in a very high degree of attenuation. The few animals with the neurological disease apparently inflicted by the mutants with AUG-lacking insertions were actually the victims of emerging virulent revertants with a restored wild-type-like arrangement of the tandem. The mutants with AUG-containing insertions proved to be virulent, although the level of virulence was modulated by the AUG context. Hence, it is the oligopyrimidine/AUG tandem as such that is essential, and this tandem should be regarded as a host-dependent *cis*-acting control element. The significance of the conserved oligopyrimidine for the template activity of poliovirus RNA also was reported to vary depending on the nature of the cells used for the preparation of translating extracts (14).

The requirements for the oligopyrimidine/AUG tandem are not only host dependent but also IRES dependent. This notion is supported by the following observations. (i) In the same cell-free system (Krebs-2 extracts), the tandem is very important for the poliovirus (18) but not TMEV (17) RNA translation, and (ii) while substitutions in the TMEV oligopyrimidine do not appear to bring about a significant adverse effect on the ability of this virus to grow in BHK-21 cells (this study and reference 17), they do interfere with the translation of the foot-and-mouth disease virus RNA in the cells of the same line (10). Also, these mutations differently affected the template activities of the TMEV (17) and foot-and-mouth disease virus (10) RNAs in the same translation system, reticulocyte lysates.

Thus, the tandem is a conditionally essential control element. What does this mean in terms of the mechanism of translation initiation? Although the exact answer is yet to come, it seems plausible that the tandem is required to stabilize the ribosome-IRES interaction when this interaction is not particularly strong because of peculiarities of the cell translation machinery. The stabilization may involve either RNA-RNA (e.g., template-rRNA; see reference 18) or RNA-protein (factor) interactions or both.

Structural requirements for a functional oligopyrimidine/AUG tandem. The conserved oligopyrimidine adjoining the TMEV IRES could be considered as being composed of two parts, the 5′-proximal and -distal portions. The proximal part consists of the pentanucleotide UUUUC and by its composition and relative location is analogous to box A of poliovirus (18); the distal part is hereinafter referred to as box A′. The box A moiety of the tandem appears to be functionally important as its destruction (in GD-20) was accompanied by a marked attenuation in mice. On the other hand, box A′ is less important for the TMEV neurovirulence, since its alterations (in GD-2 and -19) did not result in any attenuation. Nevertheless, box A′ appears to be able at least partially to substitute for box A, since preservation of the former in the box A-lacking mutant GD-20 ensured a reasonable level of neurovirulence, whereas the absence of the entire oligopyrimidine (GD-21) caused very strong attenuation. It could even be argued that

TABLE 5. Genome structure of the revertants selected from GD/PV-22 and -12

Name	Onset of paralysis		Sequence[a]	Box A-AUG distance
	Day	Dose[b]		
			1040 ↓ ... 1071 ↓	
GD/PV-22			GGUUUUCGGAUCUGUUUUAUUGUGGCUGCUUUUGG..CCUAUUAUAUUGUCAAUAUGG	129
GD/PV-22R1	10	8.2	GGUUUUCGGAUCUGUUUUAUUGUGGCUGCUUaUGG..CCUAUUAUAUUGUCAAUAUGG	24
GD/PV-22R2	11	8.2	GGUUUUCGGAUCUGUUUUAU**********..****UUAUAUUGUCAAUAUGG	26
			┌u┐	
GD/PV-22R3	15	8.2	GGUUUUCGGAUCUGUU**********..****UUAUAUUGUCAAUAUGG	23
GD/PV-12			GGUUUUCGGAUCUGUUUUAUUGUGGCUGAAAUUGG..CCUAUUAUAUUGUCAAUAUGG	129
GD/PV-12R1	16	7.5	GGUUUUCGGAUCUGUU**********..*******UAUUGUCAAUAUGG	19
GD/PV-12R2	18	7.5	GGUUUUCGaAUCUGUUU**********..**UAUUAUAUUGUCAAUAUGG	25

[a] See footnote *a* in Table 2. The deleted nucleotides are marked by asterisks.
[b] See Table 3, footnote *b*.

incompleteness of the functional substitution of box A by box A′ in GD-20 was due to a nonoptimal (5-nt-shorter) length of the oligopyrimidine/AUG spacer rather than to the intrinsic deficiency of box A′. The significance of this distance is supported by a lower virulence of the 4-nt-deletion mutant GD/Δ4-54 compared with that of GD-2 or GD-19.

The context of the AUG moiety of the tandem appears to exert some modulating effect on its functional activity in the mouse central nervous system: the less favorable the context, the more attenuated the virus. The competence of the tandem depended also on the length of the oligopyrimidine/AUG spacer. This length varied in our collection of virulent mutants and revertants in the range of 17 to 28 nt, and the viruses with borderline values of the spacer length proved to be relatively more attenuated (compare GD/Δ4-54 and GD-40R4 in Tables 1 and 4).

The tandem retains its function, as judged by the neurovirulence of appropriate mutants, even when its individual components (the oligopyrimidine or AUG) are made completely double stranded. This contrasts sharply with the structural requirements of the starting window, which should contain at least some unpaired bases (17). As the tandem and the starting window usually overlap each other, the translation-stimulating effects of duplex-destabilizing mutations around the AUG (4, 18) are more likely due to the opening of the starting window than to the activation of the tandem. The preservation of the tandem's functional activity upon duplexing of its components is compatible with the idea that the IRES-bound ribosome first makes a productive contact with the starting window, then melts the adjoining regions of the template, and, if necessary, is further stabilized on the template by tandem-mediated auxiliary interactions.

Implications for attenuation and virulence. For viruses like TMEV, whose pathogenicity requires the oligopyrimidine/AUG tandem in a host-dependent manner, the construction of highly attenuated variants may be based on the elimination of this tandem. Importantly, the potential of a virus for growth in some tissue cultures (a prerequisite for convenient vaccine production) may not necessarily be impaired by such mutations. Furthermore, the mutations can be designed in such a way that the restoration of the tandem by reversions will be of a very low probability.

Although the oligopyrimidine/AUG tandem is essential for TMEV neurovirulence, it obviously is not the only genetic determinant of the biological behavior of the virus. The capsid structure is another major factor controlling the quantitative and qualitative aspects of the viral pathogenicity (7, 8, 20, 27).

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