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Cap-Independent Translation Is Required for Starvation-Induced Differentiation in Yeast

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Our data support a model in which miR-133b functions within a feedback loop, as Pitx3 specifically induces transcription of miR-133b, and Pitx3 activity is down-regulated by miR-133b posttranscriptionally (fig. S6D). Midbrain DN function is dynamic, and such feedback circuitry has been shown to increase the robustness and speed response time and stability in the context of dynamic changes (*17*). Furthermore, we present evidence that Dicer deletion leads to the progressive loss of midbrain DNs, suggesting that miRNAs in addition to miR-133b function in these cells.

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Cap-Independent Translation Is Required for Starvation-Induced Differentiation in Yeast

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Cellular internal ribosome entry sites (IRESs) are untranslated segments of mRNA transcripts thought to initiate protein synthesis in response to environmental stresses that prevent canonical 5′ cap–dependent translation. Although numerous cellular mRNAs are proposed to have IRESs, none has a demonstrated physiological function or molecular mechanism. Here we show that seven yeast genes required for invasive growth, a developmental pathway induced by nutrient limitation, contain potent IRESs that require the initiation factor eIF4G for cap-independent translation. In contrast to the RNA structure-based activity of viral IRESs, we show that an unstructured A-rich element mediates internal initiation via recruitment of the poly(A) binding protein (Pab1) to the 5′ untranslated region (UTR) of invasive growth messages. A 5′UTR mutation that impairs IRES activity compromises invasive growth, which indicates that cap-independent translation is required for physiological adaptation to stress.

Translation initiation is a crucial point of regulation of eukaryotic gene expression, allowing cells to adapt rapidly to changing environmental conditions. In response to glucose deprivation, haploid *Saccharomyces cerevisiae* cells dramatically down-regulate translation of most cellular messages, while also exhibiting striking morphological changes leading to invasive growth (*1*, *2*). Whereas the global translational repression requires the mRNA 5′ decapping machinery (*3*), the developmental switch requires new protein synthesis, which suggests that proteins required for invasive growth might be translated by a cap-independent mechanism.

Many invasive growth genes have unusually long 5′ untranslated regions (5′UTRs) with the potential to form stable RNA secondary structures (table S1) (*4*). Furthermore, one gene required for invasive growth, *YMR181c* (*5*), is the downstream open reading frame (ORF) of a naturally occurring bicistronic cellular message (*6*), suggesting that invasive growth genes might be translated by a mechanism that depends on an internal ribosome entry site (IRES). To test whether the 5′UTRs of invasive growth genes are capable of internal translation initiation, we inserted these sequences into a firefly luciferase reporter (F-luc) containing a stable stem-loop structure [change in Gibbs free energy ($\Delta G°$) = –58 kcal/mol] at the 5′ end to inhibit scanning (*7*) and capped with a nonphysiological ApppG cap that reduces binding of the cap-binding initiation factor (eIF4E) by three orders of magnitude (*7*). With no IRES inserted, the ApppG-capped hairpin RNA is poorly translated, yielding 0.4% in vitro and 0.04% in vivo compared with an m7GpppG-capped mRNA (at 100%), with an unstructured 18-nucleotide (nt) 5′UTR (Fig. 1A) (*8*). We confirmed by Northern blots that the reporter mRNAs were stable in both extracts and cells (Fig. 1B), which ruled out differential RNA stability as a source of differences in luciferase activity. Insertion of the 5′UTR sequences from *YMR181c* or from the invasive growth genes *GPR1*, *BOI1*, *FLO8*, *NCE102*, *MSN1*, or *GIC1*, resulted in efficient translation of ApppG-capped hairpin mRNAs compared with length-matched negative control constructs containing reverse-complement 5′UTR sequences. In addition, the 5′UTRs from *TPK2*, *HMS2*, and *YEL033w* lacked IRES activity (Fig. 1C). The invasive growth cellular IRESs' activities ranged from 8 to 33% of that of the control m7GpppG-capped mRNA (table S2). We tested the IRES-containing 5′UTRs for activity in vivo by electroporating the reporter mRNAs into yeast cells to avoid any possibility of mistaking cryptic promoter or splicing activity for IRES activity. All seven 5′UTRs promoted efficient cap-independent translation in vivo (Fig. 1D). Experiments with bicistronic reporters, in which the 5′ ORF is translated via cap-dependent initiation and the 3′ ORF is efficiently translated only when an active IRES is inserted between the two ORFs, corroborated our finding that the 5′UTRs of seven invasive growth genes mediate internal translation initiation (fig. S1).

A subset of viral IRESs recruits the translation machinery by providing high-affinity internal binding sites for the translation initiation factor eIF4G (*9*). To test whether invasive growth IRESs require eIF4G, we prepared extracts genetically depleted of eIF4G (*8*). Reducing eIF4G levels to 37% decreased translation from invasive growth IRES reporters to ~25% activity (Fig. 2A), whereas translation from the eIF4G-independent cricket paralysis virus (CrPV) IRES was only slightly affected. Further depletion abolished activity (fig. S2). In cell extracts containing 9-fold overexpressed eIF4G, invasive growth IRES activity increased 10- to 20-fold (Fig. 2B), which indicated that eIF4G is limiting for IRES activity.

eIF4G is the least abundant initiation factor in yeast (*10*) and becomes unstable in nutrient-limited cells (*11*), which suggests a need for continued synthesis of eIF4G protein in glucose-starved cells in order to maintain invasive growth IRES activity. We therefore tested whether the 5′UTRs from either yeast eIF4G gene are themselves capable of internal initiation

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Fig. 1. Invasive growth genes have IRES activity. (**A**) In vitro (left) and in vivo (right) translation of m7G- and ApppG-capped poly(A)$^+$ F-luc mRNAs. For all luciferase assays, values represent the average of three independent experiments and error bars show the standard deviation (SD). m7GpppG-capped reporters were set equal to 100%. (**B**) Northern blots of F-luc mRNAs isolated from (A) after incubation in vitro (left) or in vivo (right). Relative levels of full-length F-luc RNA are indicated. (**C**) In vitro translation of F-luc reporters containing various 5′UTR inserts from *YMR181c* sequences or the reverse complement (rc) or from invasive growth genes. F-luc activity was normalized to mRNA levels as determined by Northern blot. (**D**) In vivo translation of F-luc reporter mRNAs, normalized to mRNA levels as in (C).

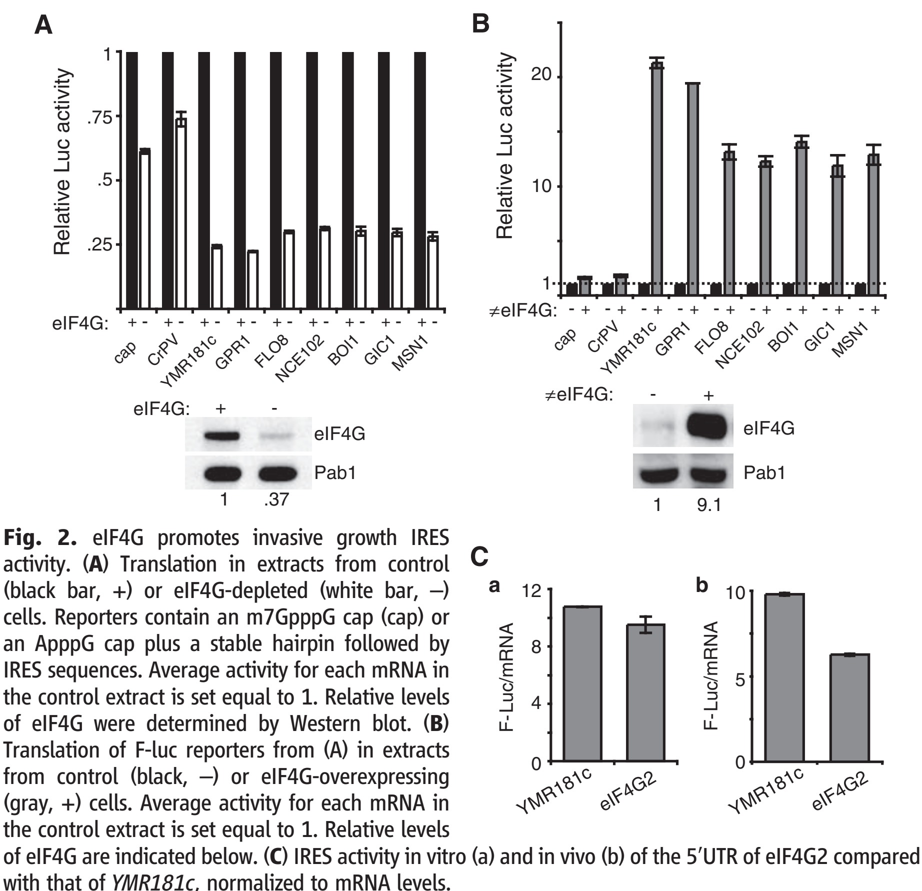

Fig. 2. eIF4G promotes invasive growth IRES activity. (**A**) Translation in extracts from control (black bar, +) or eIF4G-depleted (white bar, –) cells. Reporters contain an m7GpppG cap (cap) or an ApppG cap plus a stable hairpin followed by IRES sequences. Average activity for each mRNA in the control extract is set equal to 1. Relative levels of eIF4G were determined by Western blot. (**B**) Translation of F-luc reporters from (A) in extracts from control (black, –) or eIF4G-overexpressing (gray, +) cells. Average activity for each mRNA in the control extract is set equal to 1. Relative levels of eIF4G are indicated below. (**C**) IRES activity in vitro (a) and in vivo (b) of the 5′UTR of eIF4G2 compared with that of *YMR181c*, normalized to mRNA levels.

using the ApppG-capped hairpin reporter. The 461-nt 5′UTR of eIF4G2 showed IRES activity similar to invasive growth genes' 5′UTRs both in vitro and in vivo (Fig. 2C). Thus, cap-independent synthesis of eIF4G2 could support eIF4G-dependent IRES activity in starved cells.

Well-characterized viral IRESs use disparate strategies for cap-independent recruitment of eIF4G, but many share a requirement for the formation of stable RNA structures (*9*). We performed ribonuclease-mediated structural probing of *YMR181c*'s 298-nt 5′UTR, revealing extensive regions of secondary and tertiary structure (fig. S3). To determine which RNA elements are important for IRES activity, we constructed a series of deletion mutants. Deletion of the structured 5′-most 224 nt had little effect on IRES activity (fig. S4). The 60 nt immediately upstream of the AUG initiation codon were sufficient for robust internal translation initiation (Fig. 3A). This minimal IRES is almost completely unstructured (fig. S3), which suggests a sequence requirement for IRES activity. The minimal IRES sequence includes a polyadenosine [poly(A)] tract (12 out of 13 residues) preceding the AUG initiation codon, reminiscent of the leaders of vaccinia viral mRNAs that are capable of efficient cap-independent translation (*12*, *13*). Deletion of this poly(A) sequence from the *YMR181c* 5′UTR reduced IRES activity both in vitro and in vivo (Fig. 3A). Deletion of the A-tract had no effect on RNA integrity or stability (Fig. 3B).

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Poly(A)$^{+}$ tracts at the 3′ end stimulate eukaryotic translation through conserved poly(A)–binding proteins (PABPs) that enhance translation by at least two mechanisms: stabilizing eIF4G's interaction with the 5′ end of the mRNA through direct interactions between PABP and eIF4G and stimulating 60*S* ribosome subunit joining (*14*, *15*). In principle, both of these functions of PABP could be performed by binding to poly(A) in the 5′UTR. To test the hypothesis that the yeast PABP, Pab1, stimulates cap-independent translation of *YMR181c* specifically through binding to the 5′UTR, we examined translation of mRNAs lacking poly(A) tails. Addition of exogenous poly(A) RNA dramatically inhibited translation of an ApppG-capped hairpin mRNA containing the 5′UTR of *YMR181c*, a defect that was rescued by the addition of recombinant Pab1 (Fig. 3C). Increasing Pab1 concentration specifically enhanced translation from the wild-type *YMR181c* 5′UTR compared with the ΔA mutant IRES or an m7GpppG-capped message lacking a poly(A) tail (Fig. 3D). To test whether Pab1 binds the *YMR181c* 5′UTR directly, we performed filter-binding assays with recombinant protein. Pab1 bound tightly and specifically to the *YMR181c* RNA with an apparent dissociation constant (K_d) of 0.25 ± 0.05 μM, compared with the reverse complement control RNA (K_d = 1.52 ± 0.13 μM); deletion of the poly(A) tract eliminated specific binding (K_d = 3.29 ± 0.41 μM) (Fig. 3E).

Taken together, these data support a mechanism for *YMR181c* IRES activity requiring specific binding of Pab1 to the 5′UTR and suggest that binding of Pab1 to the 5′UTR can functionally substitute for a cap and eIF4E in recruiting eIF4G. This mechanism is not unique to *YMR181c*: The 176-nt 5′UTR from *BOI1* contains a similar AUG-proximal poly(A) tract and requires binding to Pab1 for IRES activity (fig. S5). Notably, we found that the 171-nt 5′UTR of yeast *PAB1* had robust IRES activity in vitro and in vivo (Fig. 3F), and like the invasive growth IRESs, the *PAB1* IRES was strongly eIF4G-dependent (fig. S6). Cap-independent production of both Pab1 (Fig. 3F) and eIF4G (Fig. 2C) could sustain necessary translation during prolonged periods of decreased cap-dependent initiation.

The discovery of IRESs in numerous eukaryotic regulatory genes suggests that IRES-dependent initiation plays a crucial role in cellular adaptation (*16*–*19*). To test this hypothesis directly, we determined whether the *FLO8* IRES is required for *FLO8* function by creating a yeast strain in which the *FLO8* gene was replaced with a mutant version lacking residues –60 to –11 in the 167-nt 5′UTR (*flo8 ΔIRES*). This internal deletion, which removes two poly(A) tracts (fig. S7), reduced IRES-dependent translation of a reporter gene by 60% in vitro (Fig. 4A). Deletion of nucleotides –60 to –11 from the endogenous *FLO8* locus similarly reduced Flo8 protein levels in vivo (Fig. 4B) without affecting *FLO8* mRNA levels (Fig. 4C), consistent with a requirement for IRES activity to maintain wild-type translation. No other feature of the *FLO8* gene was altered. To test whether this reduction in Flo8 is physiologically significant, we assayed the *flo8 ΔIRES* mutant strain for invasive growth and for expression of *FLO11* mRNA, which requires *FLO8* (*20*). The *flo8 ΔIRES* mutant is defective for both invasive growth (Fig. 4D) and for transcription of

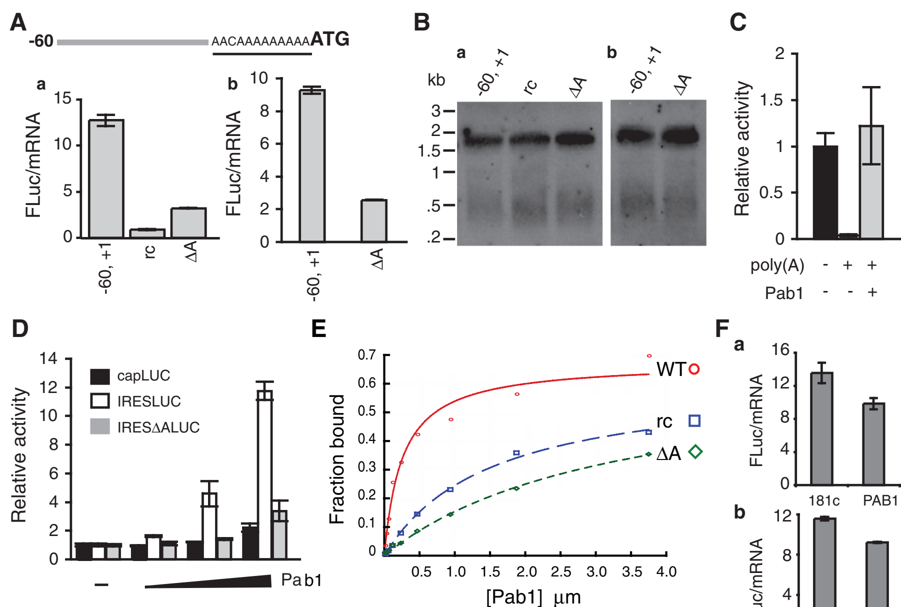

Fig. 3. An unstructured poly(A) tract mediates Pab1-dependent IRES activity. (**A**) Translation of ApppG-capped F-luc reporters containing *YMR181c* sequences or the reverse complement (rc) in yeast extracts (a) or cells (b) as described in Fig. 1. The 12-nt sequence deleted in ΔA is underlined. (**B**) Northern blots of F-luc mRNAs isolated from (A). (**C**) In vitro translation of ApppG-capped F-luc mRNA [lacking a poly(A) tail] ±100 ng exogenous poly(A) RNA ±100 ng of recombinant Pab1. The activity from the reaction without any additions was set equal to 1. Attempts to test the effects of Pab1 depletion using antibodies required high concentrations of antibody and prolonged incubations that resulted in nonspecific inactivation of extracts. (**D**) Translation of m7GpppG-capped (black), ApppG-capped + IRES (white) or ApppG-capped+IRESΔA (gray) F-luc mRNAs [lacking poly(A) tails] in the presence of 100, 200, or 400 ng rPab1 or buffer. Average activity from the reaction + buffer was set equal to 1 for each construct. (**E**) Filter-binding assay for binding of rPab1 to various *YMR181c* 5′UTR sequences showing the fraction bound for each concentration of rPab1. (**F**) Translation in vitro (a) and in vivo (b) of F-luc reporters containing 5′UTRs of *YMR181c* and *PAB1*, normalized to mRNA levels by Northern blot.

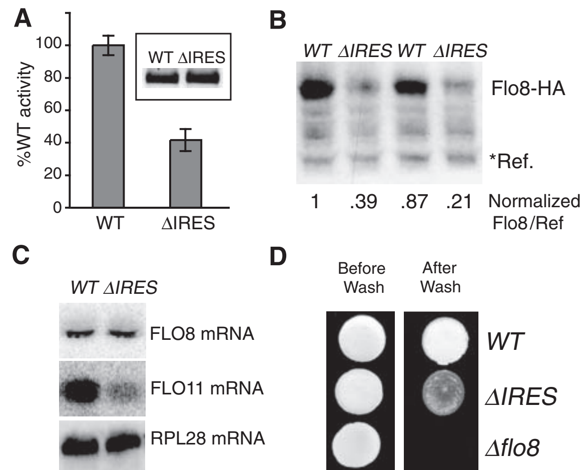

Fig. 4. IRES-dependent translation of FLO8 is required for invasive growth. (**A**) In vitro translation of ApppG-capped F-luc mRNAs containing the full-length 5′UTR from *FLO8* wild-type (WT) or a deletion mutant lacking nucleotides –60 to –11(Δ), normalized to mRNA levels by Northern blot (inset). (**B**) Western blot of Flo8 from two independent isolates each of WT *FLO8* or –60 to –11Δ mutant strains containing a C-terminal hemagglutinin (HA) epitope tag. The reference band (*Ref.) is a cross-reacting protein detected in untagged strains. Relative levels of Flo8-HA are indicated below. (**C**) Northern blots of RNA isolated from the extracts prepared in (B). (**D**) Invasive growth assay for WT, *Δflo8*, and –60 to –11Δ strains. Portions of the cultures assayed in (B) and (C) were spotted onto rich medium and photographed after growth at 30°C. Invasive growth was photographed after washing the same plate under a gentle stream of water.

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FLO11 (Fig. 4C). These data strongly argue for a physiological requirement for IRES-dependent translation in yeast invasive growth. Our findings demonstrate a direct connection between IRES activity and cellular differentiation and suggest a coherent molecular mechanism for internal initiation on cellular messages that may be conserved in higher eukaryotes.

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Strand-Biased Spreading of Mutations During Somatic Hypermutation

Shyam Unniraman and David G. Schatz*

Somatic hypermutation (SHM) is a major means by which diversity is achieved in antibody genes, and it is initiated by the deamination of cytosines to uracils in DNA by activation-induced deaminase (AID). However, the process that leads from these initiating deamination events to mutations at other residues remains poorly understood. We demonstrate that a single cytosine on the top (nontemplate) strand is sufficient to recruit AID and lead to mutations of upstream and downstream A/T residues. In contrast, the targeting of cytosines on the bottom strand by AID does not lead to substantial mutation of neighboring residues. This strand asymmetry is eliminated in mice deficient in mismatch repair, indicating that the error-prone mismatch repair machinery preferentially targets top-strand uracils in a way that promotes SHM during the antibody response.

During an immune response, B cells located in germinal centers of the lymph nodes and spleen mutate the variable region of their immunoglobulin (Ig) genes at high rates by the process of somatic hypermutation (SHM). SHM is critical for the generation of high-affinity antibodies and efficient immune responses (*1*). The reaction is initiated by the deamination of C to U in Ig genes by activation-induced deaminase (AID) (Fig. 1A) (*2*, *3*). These U's are detected either by uracil DNA glycosylase (UNG) and processed by the base excision repair pathway (*4*) or by the Msh2/Msh6 dimer and processed by the mismatch repair pathway (*5–11*) (Fig. 1A). Aberrant repair by these pathways in conjunction with error-prone polymerases (*12*), especially polymerase η (*13–16*), leads to a characteristic pattern of mutations (Fig. 1A) in which A residues are targeted approximately twice as frequently as T residues on the nontranscribed top strand (*17*). The origin of this asymmetry remains unknown. Furthermore, it has not been possible to link particular A/T mutations to the deamination of a defined C residue, leaving several central questions about SHM unanswered: (i) Do top- and bottom-strand deamination events lead to similar or different outcomes? (ii) Can a deamination event lead to A/T mutations upstream and downstream of it, or do mutations spread in only one direction? (iii) Over what distance can mutations spread from a single deamination event?

To address these issues, transgenic mice were generated with SHM substrates that contained a 100–base pair (bp) region devoid of or with only a single C residue at which the reaction could initiate (Fig. 1B) (*18*). Four variants of the parental Vκ167/PEPS Igκ transgene (*19*) were generated that differed only in a stretch of A/T nucleotides inserted near the 3′ end of the variable region (Fig. 1B): The transgene TgA contained five repeats of a 20-bp region consisting exclusively of A/T nucleotides, in a sequence chosen to maximize the occurrence of hotspots for SHM (*20*) (Fig. 1B and fig. S1). The transgene variant TgT contained the same 100 bp inserted in the reverse orientation. Because the entire 100-nucleotide (nt) stretch consisted of 50 A and 50 T residues, the overall sequence composition of TgA and TgT was identical. The transgenes TgG and TgC are identical to TgA and TgT, respectively, with the exception that they contain a single C:G (TgC) or G:C (TgG) base pair in the central repeat (Fig. 1B).

The copy number and expression level of the founder lines for each transgene showed large variation (*18*), leading us to select multiple lines of each transgene for further analysis (Table 1 and fig. S2). B cells were isolated from Peyer's patches of aged mice, and DNA was prepared from actively hypermutating germinal-center B cells ($B220^+CD19^+GL7^+$) and nonmutating control nongerminal-center B cells ($B220^+CD19^+GL7^-$). A 513-bp stretch encompassing most of the VJ region of the transgenic Ig gene, including the A/T tract, was analyzed for mutations (Figs. 1 and 2). 4.3 to 6.8% of the sequences from the $GL7^+$ samples were mutated (Table 1), with most mutated sequences possessing only a single mutation. Mutations observed in different lines of the same transgene showed similar patterns and were pooled and presented together (fig. S3 and table S1). The region outside of the A/T tract showed comparable mutations between the different constructs (Fig. 2A). We also sequenced the Ig heavy chain Jh4 intron from the same DNA samples to assess mutations in a region of the genome expected to be heavily mutated (*18*). B cells from mice harboring different transgenes had comparable levels of mutations in the Jh4 intron (fig. S4).

Analysis of the TgA and TgT lines showed that the mutation machinery was largely excluded from the A/T tract (Fig. 2B). The number of mutations was significantly lower than predicted by the mutability index of the region (P = 0.0002 and 0.0016 for TgA and TgT, respectively). The few mutations that were seen occurred in the peripheral portions of the A/T tract, up to 18 or 29 bp from the nearest C residue in the 5′ or 3′ flanks, respectively (Fig. 2B); thus, we presume that they arose from the deamination of C residues in the flanking regions. The complete protection of the central portion of the A/T tract in TgA

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