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Genetics

Developmentally excised DNA sequences in *Euplotes crassus* capable of forming G quartets

(site-specific recombination/DNA rearrangement/telomeric repeats/ciliated protozoa)

LAWRENCE A. KLOBUTCHER

Department of Biochemistry, University of Connecticut Health Center, Farmington, CT 06030

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ABSTRACT **Tens of thousands of DNA segments are eliminated by DNA breakage and rejoining events during the formation of a new macronucleus in the hypotrichous ciliated protozoan *Euplotes crassus*. This study presents evidence for a class of eliminated sequences referred to as telomeric-repeat-like internal eliminated sequences (TelIESs). TelIESs are shorter (<50 bp) than most previously characterized IESs and their DNA sequences resemble the telomeric repeat sequences of the organism. The TelIESs are excised during the developmental period of chromosome fragmentation/telomere addition, which is later than previously characterized IESs. Additional studies demonstrate that oligonucleotides representing the TelIESs are, like telomeric repeats, capable of forming G-quartet structures *in vitro*.**

After the sexual phase of its life cycle, the hypotrichous ciliated protozoan *Euplotes crassus* transforms a mitotic copy of its transcriptionally inactive micronucleus into a macronucleus with a genome consisting of linear gene-sized DNA molecules (average size, ≈2 kbp; for review, see refs. 1 and 2). The development of a new macronucleus involves an extensive rearrangement of the precursor micronuclear genome and occurs over a period of ≈100 hr. It begins with a period of DNA replication (25–55 hr after mating is initiated) that results in the formation of polytene chromosomes. The chromosomes are then fragmented to yield the linear macronuclear DNA molecules (55–60 hr) and repeats of the octanucleotide 5′-G_4T_4-3′ are added to their ends, which serve as telomeres. The telomeric repeats are initially added in an extended form (3, 4) and subsequently trimmed to the length characteristic of the mature macronucleus (5). The final stage of macronuclear development involves additional rounds of DNA replication in the absence of cell division that result in the mature macronucleus.

During macronuclear development in *E. crassus* and other ciliates, large numbers of interstitial segments of DNA are excised from the micronuclear genome with rejoining of the flanking sequences (for review, see refs. 2, 6, and 7). Two classes of DNA sequences involved in such breakage and rejoining, or splicing, events have been defined in *E. crassus*. (*i*) Two related families of transposable elements, Tec1 and Tec2, exist (≈20,000 copies of each element) in the micronuclear genome and are all excised during macronuclear development (8–10). (*ii*) The second class consists of short (31–374 bp) unique segments of DNA that have been termed "internal eliminated sequences" (IESs; ref. 8).

E. crassus IESs and Tec elements display a number of similarities. Both are bounded by a direct repeat of the dinucleotide 5′-TA-3′, and excision has been shown to be precise such that one copy of the 5′-TA-3′ repeat is retained in the resulting macronuclear DNA molecule (11). The excision process occurs during the polytene chromosome stage of development, with the Tec elements being excised early in the polytenization process (25–30 hr; ref. 9), while the larger IESs are removed later (45–50 hr; ref. 4). Once excised, Tec elements and IESs assume free circular forms with an unusual heteroduplex junction region (12, 13). These similarities have led to the suggestion that IESs represent degenerate or nonautonomous forms of the Tec elements (see ref. 6 and references therein).

The current study presents evidence for a class of IESs in *E. crassus* termed telomeric-repeat-like IESs* (TelIESs). These IESs differ from the well studied IESs in that they are short (<50 bp), their sequences resemble the telomeric repeats of macronuclear DNA molecules, and they are excised later than the larger IESs. Like telomeric repeats, oligonucleotides representing these IESs are shown to form G-quartet structures *in vitro*.

G quartets represent an alternative nucleic acid structure in which four guanine residues interact through hydrogen bonding to form a square-planar array (14–16). *In vitro*, G quartets have been found to form in sequences that have blocks of guanine residues, such as telomeric repeat sequences (15, 16), the immunoglobulin heavy chain switch regions (14), and retroviral dimerization domains (17, 18). Sequences with multiple blocks of guanine residues can form intramolecular G-quartet structures (15). In addition, DNA segments with only one or two blocks of guanine residues can form G quartets by intermolecular interactions, with the strands in either parallel or antiparallel orientation (e.g., refs. 14, 16, and 19–21). Although the *in vivo* significance of G quartets remains an open question, recent studies have identified proteins that foster the formation of G quartets (e.g., ref. 22) or that cleave DNA in the G-quartet conformation (23). The potential significance of the G-quartet forming ability of the TelIESs is discussed in relation to these findings.

MATERIALS AND METHODS

Cell Lines, Cell Culture, and DNA Isolation. *E. crassus* culture and matings were as described (24), with minor modifications (4, 11). Strains used for matings were either ST9 and ST11 or CC100 and 14 (9, 11, 25). Developing macronuclei were purified and DNA was isolated as described (5, 24). Characterized preparations of DNA from developing macronuclei 52 and 57 hr after mating (26) were kindly provided by J. Vermeesch and C. Price (University of Nebraska).

Synthetic Oligonucleotides and Polymerase Chain Reaction (PCR). Synthetic oligonucleotides were purchased from the Biotechnology Center at the University of Connecticut (Storrs). Oligonucleotides used in G-quartet studies were further purified by electrophoresis through 10% polyacrylamide/7 M urea gels, followed by elution into TE buffer (10 mM Tris·HCl, pH 7.4/1 mM EDTA). Oligonucleotides V14, V2MG, and V2MT have been described (11). The sequences of other oligonucleotides used in these studies are as follows:

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Abbreviations: IES, internal eliminated sequence; TelIES, telomeric-repeat-like IES; DMS, dimethyl sulfate.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M28500).

V14, 5′-GGGTAAAAGTCTGTAAAGCTTATAAAAC-3′; V2IES, 5′-TAGTTAAGGGGTTGGGGTTTTGTTTTAGGTGTA-3′; sV2IES, 5′-TAGTTAAGGGGTTGGGGTTTTGTTT-3′; V3IES, 5′-TATATGGGATTGTGGGGTTTTAGGAGATAAAAGGATTTAGTATA-3′; sV3IES, 5′-TATATGGGATTGTGGGGTTTTAGGAGA-3′; LV3, 5′-CTAACTCTGGTTTAGTGGCT-3′, V3/VX, 5′-GGATAGACCTTCTTTCTGGC-3′.

PCR analyses (27) were performed by using a GeneAmp PCR kit (Perkin–Elmer/Cetus) but with *Taq* I polymerase and reaction buffer purchased from Life Technologies (Grand Island, NY). Amplifications were carried out for 25–30 cycles by using a model PTC-150 MiniCycler (MJ Research, Cambridge, MA). For the 31-bp V2 IES, an amplification cycle consisted of 75 sec at 94°C, 75 sec at 45°C, and 75 sec at 72°C. The amplification cycle for the 42-bp V3 IES was identical, except that the annealing temperature was raised to 50°C.

G-Quartet Reactions. For G-quartet analyses, the oligonucleotides were 5′-end-labeled by using [γ-^{32}P]ATP and T4 polynucleotide kinase (28), desalted by passage through a Biospin chromatography column (Bio-Rad), and when necessary, concentrated by lyophilization.

To assess G-quartet formation, oligonucleotides were prepared at 20 nM or 10 μM in the presence of either 50 mM or 200 mM NaCl. The samples were then boiled for 5 min and incubated at 37°C for 16–24 hr. The G-quartet reactions were analyzed by electrophoresis on 10 or 12% nondenaturing polyacrylamide gels as described (15), except that 50 mM NaCl was included in the gel and running buffer and the gels were electrophoresed in a cold room at 4°C.

Dimethyl Sulfate (DMS) Methylation Protection. For DMS methylation protection, G-quartet forms of the V2IES oligonucleotide were generated as described above, but 30 mM Hepes (pH 7.6) and 1 mM EDTA were included in the reaction mixture. For the V3IES oligonucleotide, G-quartet reactions were performed as described above and the products were separated by electrophoresis. The G-quartet forms were excised from the gel and eluted into 250 μl of 30 mM Hepes, pH 7.6/1 mM EDTA/50 mM or 200 mM NaCl. DMS methylation (22), piperidine cleavage (29), and electrophoretic analysis of the products (29) were then carried out essentially as described. Quantitation of radioactivity on the sequencing gels was performed by using a Betascope 603 analyzer (Betagen, Waltham, MA) or an InstantImager 2024 (Packard).

RESULTS

Small IESs That Resemble Telomeric Repeats Are Excised Late in Development. Most of the IESs that have been identified in *E. crassus* are >100 bp long and extremely rich in A·T base pairs (ref. 8 and unpublished results). Two IESs have been identified in a cloned segment of micronuclear DNA (clone LEMICV, Fig. 1), however, that differ in both respects, a 31-bp IES and a 42-bp IES that reside in the precursors of two macronuclear DNA molecules referred to as V2 and V3, respectively (ref. 8; C. Hale, M. Jacobs, and L.A.K., unpublished results). Aside from being small and having a higher G + C content than the larger IESs, these two IESs showed an absolute strand asymmetry in regard to the presence of guanine and cytosine residues (Fig. 1*B*). Moreover, the sequences of both of these IESs resembled the 5′-G_4T_4-3′ telomeric repeat sequences of hypotrich macronuclear DNA molecules (30) and included perfect copies of a telomeric repeat located 13 bp from their ends (Fig. 1*B*).

The two small IESs also differed from the larger IESs in their timing of excision during macronuclear development. A previous study (11) provided preliminary evidence that the 31-bp V2 IES was not excised at 50 hr of macronuclear development, a time at which the larger IESs have already been removed (4). To determine the time of excision for the TelIESs more precisely, PCR experiments on DNA isolated from developing macronuclei at various times of development were carried out. PCR primers were chosen that flank the TelIESs in their micronuclear configurations such that different-sized PCR products were generated from the substrate DNA, depending on whether the IES was present or absent (Fig. 1*A*). DNAs were analyzed from developing macronuclei 52, 57, and 60 hr after mixing cells of opposite mating types. Previous Southern blot hybridization analyses (ref. 26 and data not shown) of these DNA preparations indicated (*i*) the 52-hr DNA preparation represented the late polytene chromosome stage of development and that a large IES, the 374-bp V1 IES, had been excised by this time, (*ii*) the 57-hr DNA represented developing macronuclei in the midst of the chromosome fragmentation/telomere addition process, such that some of the DNA was still in chromosomal form while other DNA molecules had undergone fragmentation and telomere addition, and (*iii*) the 60-hr DNA preparation represented developing macronuclei that had completed the chromosome fragmentation/telomere addition process.

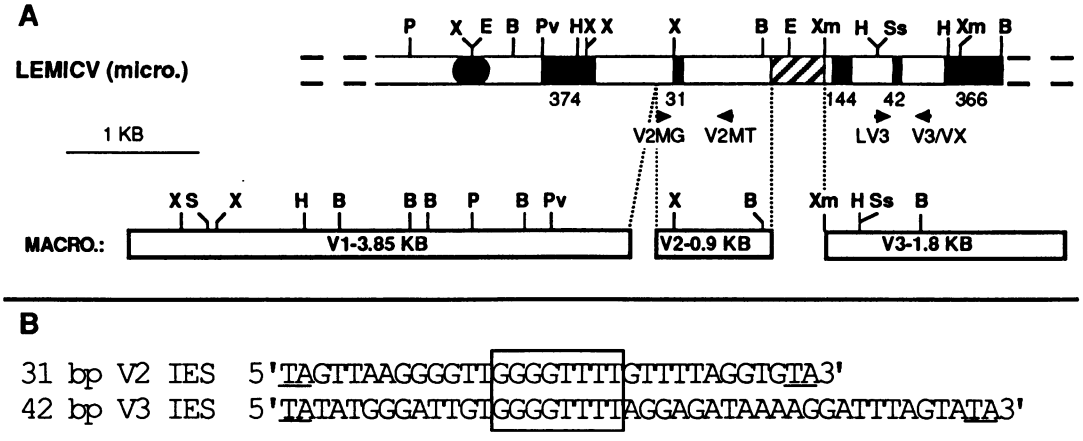

FIG. 1. (*A*) Restriction maps of a portion of the micronuclear DNA insert in clone LEMICV (8) and the three macronuclear DNA molecules derived from this region (V1, V2, and V3). In the micronuclear DNA, regions that give rise to macronuclear DNA are shown as open boxes, IESs are shown as solid boxes with their sizes indicated, and an eliminated spacer region is a cross-hatched box. Arrowheads denote the locations and direction of DNA synthesis of oligonucleotides used as PCR primers. B, *Bgl* II; E, *Eco*RI; H, *Hin*dIII; P, *Pst* I; Pv, *Pvu* II; S, *Sal* I; Ss, *Sst* I; X, *Xba* I; Xm, *Xmn* I. (*B*) Sequences of the 31-bp V2 IES and 42-bp V3 IES, including both terminal 5′-TA-3′ direct repeats (underlined). The perfect telomeric repeat within each IES is boxed.

When oligonucleotides V2MG and V2MT (Fig. 1*A*) were used to amplify the segment of DNA containing the 31-bp V2 IES, the 52-hr preparation produced almost exclusively 400-bp PCR products, which is the expected size of the product containing the IES (IES^+ form; Fig. 2*A*). The 57-hr DNA preparation produced similar amounts of the IES^+ form and a 369-bp product representing the IES^- form, and the 60-hr DNA preparation produced primarily IES^- forms. These results indicate that the 31-bp IES is excised during the period when chromosome fragmentation/telomere addition is occurring, which is 5–10 hr later than the time when the larger IESs are excised (4).

Similar results were obtained when oligonucleotide primers LV3 and V3/VX (Fig. 1*A*) were used to amplify the 42-bp IES-containing region (Fig. 2*B*). However, in this case, a PCR product representing the IES^- form was already detectable at 52 hr of development, suggesting that the excision of this IES began somewhat prior to the excision of the 31-bp IES. It is likely that these slightly differing results are an artifact of the PCR procedure used to examine IES removal. In a control PCR experiment using mixtures of cloned DNAs with and without the IESs as PCR substrates, the IES^- form of the 42-bp IES amplified more efficiently than the IES^+ form, but no such effect was observed for the 31-bp IES (data not shown). Thus, the small amount of contaminating old macronuclear DNA molecules (lacking IESs) present in the 52-hr DNA preparation could be responsible for the IES^- PCR products observed at this time.

TelIESs Form G-Quartet Structures. The observation that the TelIES are excised during the period of chromosome fragmentation/telomere addition suggested that their excision may be somehow related to their resemblance of telomeres. One feature of the G-rich strand of true telomeric DNA is that it has the ability to form G-quartet structures *in vitro* (15, 16, 19–21). As such an alternative DNA structure provides a potential means of recognition for DNA segments destined for excision, the ability of the TelIESs to form G quartets was assessed. Oligonucleotides representing the entire 31-bp V2 IES and the 43-bp V3 IES (oligonucleotides V2IES and V3IES, respectively), including both terminal 5′-TA-3′ direct repeats, were synthesized. These oligonucleotides were then incubated at 37°C in the presence of 200 mM NaCl, conditions that favor the formation of G-quartet structures. The reactions were analyzed by native polyacrylamide gel electrophoresis, along with control samples that had been boiled immediately prior to electrophoresis (Fig. 3). At 20 nM oligonucleotide, a small amount of a slower-migrating species was seen for the V2IES oligonucleotide. At 10 μM oligonucleotide, essentially all of the V2IES was observed in the slow migrating form. For the V3IES oligonucleotide, no slower-migrating species was observed at 20 nM oligonucleotide, but ≈50% of the oligonucleotide was observed as a slower-migrating species when incubation was carried out at 10 μM oligonucleotide. A control oligonucleotide lacking a run of four guanine residues, V14, showed no difference in migration under the experimental conditions.

The observed slower migrating species for the TelIES oligonucleotides provided an indication that the oligonucleotides were capable of forming alternative structures and were consistent with the formation of G quartets. The concentration dependence suggests that the slower-migrating species were the result of intermolecular interactions (see refs. 14, 19, and 22).

To determine whether the slow-migrating species observed in the above analyses did indeed represent G-quartet structures, DMS methylation protection experiments were carried out (guanine residues involved in G-quartet structures are not accessible to DMS methylation; refs. 14 and 15). The V2IES was incubated at 10 μM in the presence of 50 mM or 200 mM NaCl and then subjected to DMS methylation and cleavage at methylated guanine residues. Analysis of the products on sequencing gels indicated that the two blocks of four contiguous guanine residues (G8 to G11 and G14 to G17) in the V2IES oligonucleotide were ≈80% protected from methylation relative to the control (Fig. 4; position G22, which undergoes no obvious methylation protection, was used as a loading standard).

A similar DMS methylation analysis of the V3IES oligonucleotide indicated that the block of four guanine residues (G14 to G17) in this IES was efficiently protected from methylation

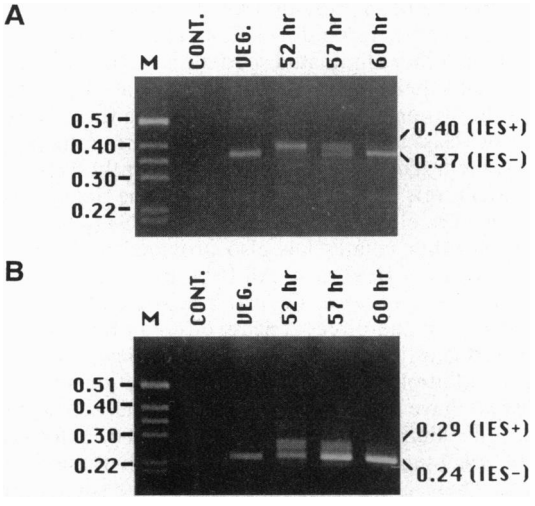

FIG. 2. PCR analyses of the time of excision of TelIESs. Agarose gels are shown that contain the PCR products obtained from total vegetative cell DNA (lane VEG.; total cell DNA is primarily macronuclear DNA) and DNAs isolated from developing macronuclei 52, 57, and 60 hr after mating was initiated (lanes 52 hr, 57 hr, and 60 hr). Size markers (lane M) and a PCR control (lane CONT.), consisting of products from a reaction prepared and run without substrate DNA, are also shown. (*A*) PCR of the genomic regions containing the 31-bp V2 IES with oligonucleotide primers V2MG and V2MT. (*B*) PCR of the genomic regions containing the 42-bp V3 IES with oligonucleotide primers V3/VX and LV3. Sizes of DNA fragments are indicated in kbp, as are the positions of PCR products containing (IES^+) and lacking (IES^-) the IES.

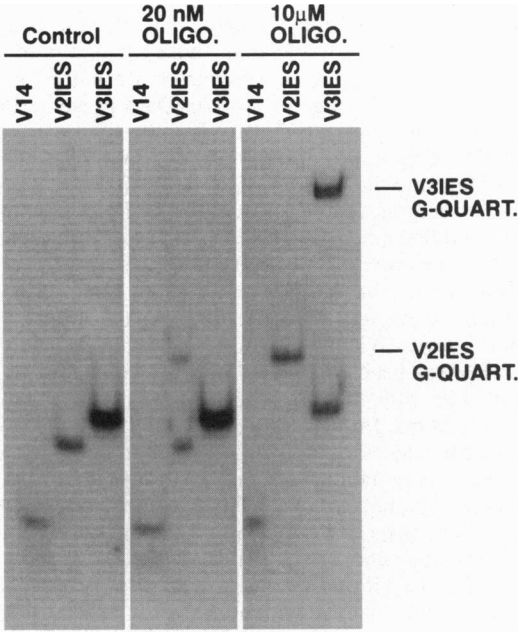

FIG. 3. TelIES oligonucleotides form G-quartet structures. An autoradiograph of a nondenaturing polyacrylamide gel is shown containing oligonucleotides V14, V2IES, and V3IES. The oligonucleotides were incubated at 20 nM or 10 μM in 200 mM NaCl at 37°C for 17 hr. Control reaction mixtures were boiled prior to loading. Bands representing putative G-quartet forms are indicated.

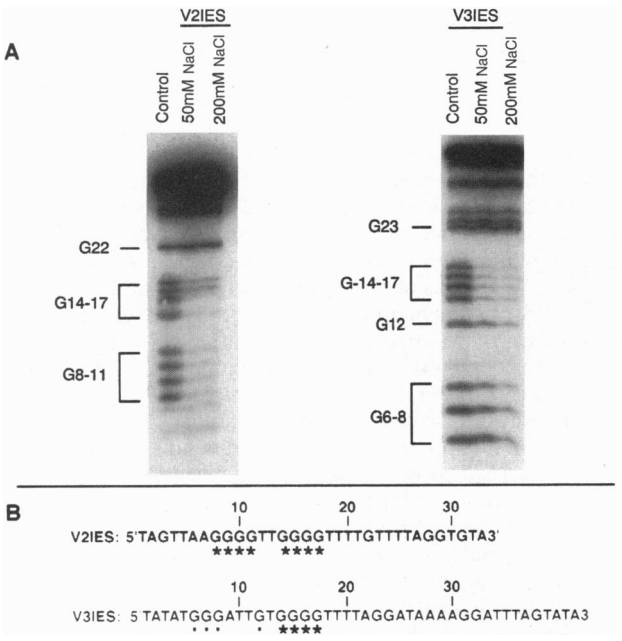

FIG. 4. DMS methylation protection analysis. (*A*) Autoradiographs of sequencing gels of DMS methylation protection products of the V2IES and the V3IES oligonucleotides after incubation in 50 mM or 200 mM NaCl and after boiling (lane Control). Positions of selected guanine residues are indicated. (*B*) Sequences of the V2IES and the V3IES oligonucleotides with guanine residues that were protected from DMS methylation indicated by asterisks. Small asterisks below the V3IES sequence denote positions that were less strongly protected from DMS methylation.

(90% protection) in the sample incubated at 200 mM NaCl (Fig. 4). In addition, the guanine residues at positions 6–8 and at position 12 were also protected from methylation, but to a lesser degree (68–76% protection at 200 mM NaCl; Fig. 4). Even less protection of these residues was observed when G quartets were formed in 50 mM NaCl. There are at least two possible explanations for the different degrees of protection observed. Residues 6–8 and 12 might be involved in G-quartet structures that are less stable than those formed by residues 14–17 or different types of G-quartet structures may be formed by the V3IES oligonucleotide. Data presented in the following section support the latter hypothesis.

The TelIES Oligonucleotides Form Intermolecular G Quartets. To determine the number of DNA strands present in the G-quartet structures, the strategies described by Sundquist and Klug (16) and Sen and Gilbert (19) were used. Two additional oligonucleotides were synthesized (sV2IES and sV3IES) that were identical to the V2IES and V3IES oligonucleotides, except that 8 bases and 17 bases, respectively, were omitted from their 3′ ends. When a mixture of oligonucleotides V2IES and sV2IES was used to form G quartets, three bands were observed (Fig. 5*A*). Two species comigrated with the G-quartet structures formed individually by the V2IES and sV2IES oligonucleotides. A third species had an intermediate mobility and presumably represents heterodimeric G-quartet structures formed between the two oligonucleotides (V2IES–sV2IES). Thus, the results indicated that the V2IES formed dimeric intermolecular G-quartet structures.

The results of a similar analysis on the V3IES were somewhat more complicated. A mixture of the V3IES and sV3IES oligonucleotides produced three major G-quartet species of intermediate mobility (Fig. 5*B*), which was consistent with the majority of V3IES G quartets, representing tetramers. However, both species of homotetramers appear to be underrepresented, and a number of additional minor bands of intermediate mobility were observed on longer exposure. This

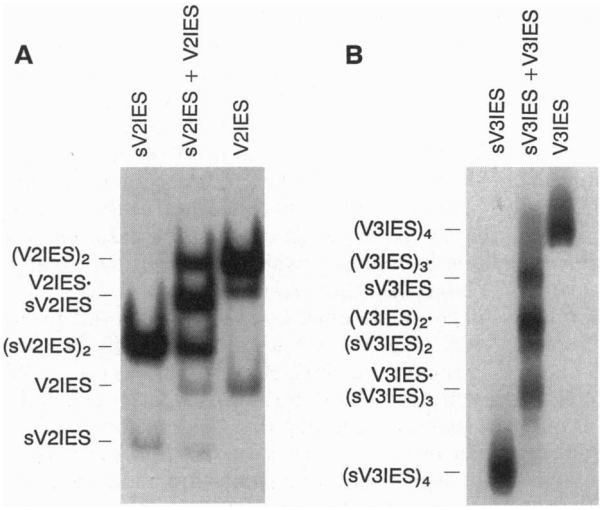

FIG. 5. Multimeric nature of the TelIES G-quartet structures. (*A*) A nondenaturing polyacrylamide gel is shown displaying the G-quartet structures formed by the V2IES oligonucleotide (lane V2IES), the sV2IES oligonucleotide (lane sV2IES), and a mixture of the two oligonucleotides (sV2IES + V2IES). The positions of the oligonucleotides in the non-G-quartet (monomeric) configuration are indicated, as are the deduced dimeric G-quartet forms. (*B*) G-quartet structures formed by the V3IES and sV3IES oligonucleotides with conventions as described above. Note that the non-G-quartet forms have been electrophoresed off the gel in the V3IES/sV3IES analysis.

result and the DMS methylation analysis suggested that there was heterogeneity in the types of G-quartet structures formed by the V3IES. This heterogeneity could consist of a mixture of parallel and antiparallel structures, dimeric and tetrameric forms, or possibly alternatively aligned strands, as suggested by Fang and Cech (22).

DISCUSSION

TelIESs Represent a Class of Developmentally Excised DNA. Data have been presented for the existence of a class of IESs in *E. crassus*, termed TelIESs. These IESs are distinguished from other eliminated sequences by their short length (<50 bp) and their unusual telomeric repeat-like sequences. Perhaps the strongest reason for considering these IESs to be in a distinct class is that they are excised during the period of chromosome fragmentation and telomere synthesis (≈55 hr of development; refs. 3 and 4), which is after the time of excision of both the Tec elements and the larger IESs (4, 9).

Work on other ciliates has also provided indications that multiple classes of IESs exist. All IESs are bordered by direct repeats in *Oxytricha nova, Oxytricha fallax*, and *Tetrahymena thermophila*, but the direct repeats differ in both length and sequence, suggesting heterogeneity (for review, see refs. 2, 6, and 7). In addition, deletion and transformation studies in *T. thermophila* have demonstrated that purine-rich sequences flanking one eliminated DNA segment are crucial for excision (31) but other excised DNA segments lack these sequence elements. These observations have led to the suggestion that a single basic excision machinery exists in the cell but that different accessory proteins interact with the different classes of eliminated DNA to target their excision (7).

It has been suggested that IESs represent degenerate forms of transposable elements (see refs. 6 and references therein). In *E. crassus*, the existence of such a relationship is strengthened by the numerous similarities in the excision processes of the larger IESs and the Tec elements (see Introduction). It is similarly possible that TelIESs represent degenerate forms of a larger repetitive element in the micronuclear genome. Repetitive elements associated with telomeric repeat sequences have been found in a number of ciliates (32–35), including the

developmentally excised TBE transposons of *O. fallax* and *Oxytricha trifallax* that posses 17 bp of telomeric repeat sequence at their termini (32, 33). It will clearly be of interest to determine whether similar telomere-associated repetitive elements exist in *E. crassus*. Knecht and Klobutcher (40) have found that ≈1000 copies of telomeric repeat sequence tracts of at least 17 bp exist within the micronuclear genome of *E. crassus*. These internal telomeric repeat tracts could represent the postulated telomere-bearing elements and/or additional TelIESs.

Potential Involvement of G Quartets in the TelIES Excision Process. The observation that the TelIESs are excised during the period of *de novo* telomere synthesis and the resemblance of TelIESs to telomeric repeats suggest that their excision may be mechanistically coupled to some aspect of telomere synthesis, structure, or function. The data presented indicate that the G-rich strands of the TelIESs are capable, like telomeres, of forming G-quartet structures *in vitro* (15, 16, 19). The G-quartet forms observed for the 31-bp V2 IES are dimeric structures, whereas the 42-bp IES forms primarily tetraplex G quartets. It is also worth noting that the TelIESs represent a second example of sequences involved in DNA rearrangement that can form G-quartet structures, as the immunoglobulin heavy chain class switching regions have been shown (14) to form such structures.

How might the ability to form G quartets be involved in the excision of TelIESs? One interesting possibility derives from the observation that yeast possess an enzymatic activity that recognizes G quartets and cleaves the DNA 17–23 bases on the 5′ side of such structures (23). The gene encoding this activity has recently been identified (36) and corresponds to a previously identified gene [variously referred to as *KEM1*, *SEP1*, *DST2*, *XRN1*, and *RAR5*, see ref. 36 for references] required for meiosis. Moreover, a number of additional activities for the protein encoded by this gene have been identified, including the ability to promote DNA strand transfer (37, 38). If a similar protein is produced in *E. crassus*, it could be involved in the excision of TelIESs. Studies of other site-specific recombination and transposition processes indicate that many can be viewed as a two-step process in which a free 3′-OH terminus is generated by strand cleavage and is subsequently transferred to a new target DNA site (39). In the current situation, a G-quartet-specific endonuclease might cleave at one end of a TelIES (the telomeric repeat located 13 bp from the 5′ ends of the TelIESs may be significant in this regard; Fig. 1*B*) to generate a free 3′-OH that would subsequently attack a phosphodiester bond at the opposite end of the IES to effect excision of the IES and joining of the flanking sequences.

The above model requires that G-quartet structures form *in vivo*. One aspect of *in vivo* G-quartet formation that appears problematical is that high DNA concentrations are required for G-quartet formation *in vitro*. For the TelIESs, this may not be a problem, as the G-quartet structures would presumably form at the polytene chromosome stage of macronuclear development when a large number of DNA strands are in close proximity. A second potential problem is that G-quartet formation has been observed only with single-stranded nucleic acid, so that local denaturing of the TelIESs in the cell would be required. DNA supercoiling might facilitate such local unwinding of the helix. Alternatively, proteins that enhance the formation of G-quartet structures have been identified (e.g., ref. 22), and they, or other proteins, might play a role in facilitating local unwinding. It should also be pointed out that local unwinding of the DNA is almost certainly involved in the excision of the TelIESs whether or not G quartets are involved, as the small sizes of these IESs makes it quite unlikely that the two ends could interact while in a duplex configuration. Determining whether G-rich DNA can form G quartets *in vivo* would clearly help resolve these problems. The ability to generate large numbers of *E. crassus* cells synchronously proceeding through macronuclear development may be beneficial in regard to addressing *in vivo* G-quartet formation for the TelIESs.

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