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Organization of telomeric and subtelomeric chromatin in the higher plant *Nicotiana tabacum*

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Abstract We have examined the structure and chromatin organization of telomeres in *Nicotiana tabacum*. In tobacco the blocks of simple telomeric repeats $(TTTAGGG)_n$ are many times larger than in other plants, e.g., *Arabidopsis thaliana* or tomato. They are resolved as multiple fragments 60–160 kb in size (in most cases 90–130 kb) on pulsed-field gel electrophoresis (PFGE) of restriction endonuclease-digested DNA. The major subtelomeric repeat of the HRS60 family forms large homogeneous blocks of a basic 180 bp motif having comparable lengths. Micrococcal nuclease (MNase) cleaves tobacco telomeric chromatin into subunits with a short repeat length of 157±5 bp; the subtelomeric heterochromatin characterized by tandemly repeated sequences of the HRS60 family is cut by MNase with a 180 bp periodicity. The monomeric and dimeric particles of telomeric and subtelomeric chromatin differ in sensitivity to MNase treatment: the telomeric particles are readily digested, producing ladders with a periodicity of 7 bp, while the subtelomeric particles appear to be rather resistant to intranucleosomal cleavage. The results presented show apparent similarities in the organization of telomeric chromatin in higher plants and mammals.

Key words Plant telomeres · Chromatin · Repetitive DNA

Introduction

In eukaryotic cells the ends of chromosomes are composed of specialized structures called telomeres containing tandemly repeated DNA. They are thought to be essential for protecting the chromosome ends from degradation and end-to-end fusion, and for maintaining precise chromosome localization within the nucleus. In most organisms telomeres are composed of simple, highly conserved repeats with a common consensus sequence $(T/A)_mG_n \cdot C_n(A/T)_m$ (for a review see Zakian 1989). A complex of less conserved arrays of repeats is usually located in an immediately proximal, subtelomeric region. At the molecular level the simple telomeric repeats have been shown to form non-B-DNA structures owing to the unusual interaction between guanine bases (Williamson et al. 1989). The protein composition of telomeres has been previously studied mainly in lower eukaryotes (Budarf and Blackburn 1986; Wright et al. 1992). In yeasts and ciliates the telomeric repeats are short and do not seem to be packed into nucleosomes. Analysis of telomeric chromatin in higher eukaryotes has been reported in rats (Makarov et al. 1993) and recently in humans (Tommerup et al. 1994). The authors have found that mammalian telomeric repeats are organized in nucleosome-like structures with an unusually short repeat length. In plants, telomeric sequences have been cloned and sequenced in *Arabidopsis thaliana* (Richards and Ausubel 1988). The terminal heptanucleotide repeat $(TTTAGGG)_n$ has been used in numerous in situ hybridization studies and it has been shown to detect chromosome ends of other plant species including tomato (Ganal et al. 1991), barley (Schwarzacher and Heslop-Harrison 1991) and tobacco (Kenton et al. 1993). The length of the telomeric repeats has been estimated to be 2–5 kb in *Arabidopsis* (Richards and Ausubel 1988) and 20–50 kb in the tomato (Ganal et al. 1991).

In this study we investigated the organization, repeat length and chromatin structure of tobacco telomeres. *Nicotiana tabacum* was chosen because the properties of tobacco chromatin have been previously studied in detail with reference to several repetitive sequences families (Fajkus et al. 1992) including the subtelomeric HRS60 family. To examine the long-range structure of tobacco telomeres we employed pulsed-field gel electrophoresis (PFGE) combined with Southern blot analysis using subtelomeric HRS60 and telomeric $(TTTAGGG)_n$ probes.

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Partial micrococcal nuclease (MNase) digestion of isolated nuclei revealed differences in the structures of telomeric and subtelomeric chromatin.

Materials and methods

Isolation of tobacco protoplasts

The procedure originally described for preparation of tomato protoplasts (Wu et al. 1992) was used with minor modifications. Pieces of fully grown young leaves with the lower epidermis peeled-off were laid on the surface of a digestion buffer {0.5 M mannitol, 20 mM 2[N-morpholino]ethanesulfonic acid (MES) pH 5.6, 1% Cellulysin (Calbiochem) and 0.05% Pectolyase Y-23 (Seishin Pharmaceutical)} in a plastic petri dish. After 2–3 h of gentle shaking at 26°C complete conversion of leaves to protoplasts was observed. Protoplasts were filtered sequentially through 80 and 30–40 μm sieves and the filtrate was centrifuged at 200 *g* at room temperature for 5 min. The sediment was suspended in a protoplast buffer (the same as the digestion buffer, but without enzymes) and the number of protoplasts was determined. After another centrifugation step, protoplasts were resuspended in the protoplast buffer to a final concentration of $2–4\times10^7$/ml. The protoplast suspension was then mixed with an equal volume of 1.5% low-melting agarose in the protoplast buffer and transferred to a mold. After solidification at 4°C, agarose blocks were transferred into ESP (0.5 M EDTA, pH 9.25, 1% Sarkosyl, 0.5 mg/ml proteinase K) and incubated at 50°C for 48 hr.

Pulsed-field gel electrophoresis

Agarose-embedded samples digested with proteinase K were washed twice in TE (10 mM TRIS-HCl, 1 mM EDTA, pH 8.0) with 0.2 mM phenylmethylsulfonyl fluoride (PMSF; Serva), and twice in TE, for 30 min each. The agarose blocks were preincubated in the appropriate restriction buffer containg BSA (0.1 mg/ml) at 4°C for 15 min and then digested in fresh restriction buffer containing 30 units of restriction enzyme for 16 h. After digestion, the samples were transferred into ES (0.5 M EDTA, pH 9.25, 1% Sarkosyl) and stored, or directly loaded onto a 1% agarose gel. Electrophoresis was carried out in a CHEF-DR II apparatus (Bio-Rad). After electrophoresis, the gel was stained with ethidium bromide, alkali-blotted onto Hybond N+ membrane (Amersham) and subsequently hybridized with subtelomeric and telomeric DNA probes.

DNA probes

The plant telomere-specific probe was generated by the polymerase chain reaction (PCR) using oligonucleotide primers T1 $(5'\text{-TTTAGGG-}3')_5$ and T2 $(5'\text{-CCCTAAA-}3')_5$. The oligonucleotides were synthesized at the Royal Botanic Gardens, Kew, Richmond (England) on a Pharmacia Gene Assembler Plus using standard cyanoethyl-phosphoramidite chemistry. Amplification was done in the absence of template DNA in a standard reaction mixture containing 50 mM KCl, 10 mM TRIS-HCl, pH 8.0, 2.0 mM $MgCl_2$, 1 unit of *Taq* polymerase (Boehringer), 200 μmol of each dNTP and 200 nM each primers T1 and T2 (Cox et al. 1993). Temperature cycling conditions were as follows: 10 cycles each of 1 min at 94°C, 30 s at 60°C and 1 min at 72°C followed by 30 cycles each of 1 min at 94°C, 30 s at 60°C, 1.5 min at 72°C, and 1 final step of 5 min at 72°C. Digoxigenin-dUTP (DIG-11-dUTP, Boehringer) supplemented with fresh *Taq* polymerase (0.5 units) was added to the reaction 10 cycles prior to the end of the program to a final concentration of 40 μmol. Approximately 100–200 ng of the heat-denatured probe was used for each hybridization. The hybridizations were performed in high stringency conditions at 68°C overnight, according to the manufacterer's (Boehringer) method for nonradioactive probe detection. The bands were visualized in a color reaction with anti-digoxigenin-alkaline phosphatase antibody conjugate using bromochloroindolyl phosphate/nitroblue tetrazolium salt as a substrate (Boehringer). A cloned dimeric fragment of highly repetitive HRS60 sequences (Fajkus et al. 1992) was used as the subtelomere-specific probe. The DNA was ^{32}P-labeled using the Megaprime kit (Amersham, England).

Analysis of chromatin structure

Nuclei of *N. tabacum* for MNase treatment were prepared from 10 g of fresh tobacco leaves according to the protocol described by Espinás and Carballo (1993). The nuclei were resuspended in a buffer containing 10 mM HEPES-NaOH, pH 7.5, 20 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 0.2 mM PMSF, 250 mM sucrose, 5 mM $MgCl_2$ and 3 mM $CaCl_2$. The nuclear suspension was divided into equal aliquots and MNase was added to a final concentration of 100 U/ml. Digestion was performed for 0, 1, 3, 10, and 30 min at 37°C. The reaction was terminated by addition of 1 vol. of solution containing 1% Sarkosyl, 0.5 M EDTA, pH 9.25, and 2 M NaCl. Samples were incubated with proteinase K (200 μg/ml) at 50°C for 1 h and extracted once with phenol/chloroform and once with chloroform. DNA was precipitated, dissolved in TE and separated either on 1.5% agarose gels or 7% polyacrylamide gels. Both denaturing (with 50% w/w urea) and non-denaturing polyacrylamide gel electrophoresis (PAGE) were performed at 60°C on a Hoefer SE 600 apparatus at 40 W in TBE buffer. After electrophoresis the gels were stained with ethidium bromide, capillary-blotted onto a Hybond N+ membrane (Amersham) and hybridized subsequently with radioactively labeled HRS60 and digoxigenin-labeled telomeric probes (see above).

Results

Tobacco chromosomes contain very long telomeric repeats

To determine the size of telomeric $(TTTAGGG)_n$ tandem repeat blocks high molecular weight tobacco DNA was digested in agarose blocks with five frequently cutting restriction enzymes that do not cleave within the repeated unit (*Alu*I, *Bst*NI, *Hae*III, *Hin*fI, *Taq*I), separated by PFGE and hybridized with the $(TTTAGGG)_n$ probe. The enzymes digested tobacco DNA into fragments with an average length of less than 5 kb (data not shown). It is evident from the data shown in Fig. 1 that all restriction enzymes used produced essentially the same hybridization pattern, suggesting that the bands represent blocks of monotonously repeated DNA without any longer intervening sequences. The maximum intensity of the signal appeared in the region of 90–130 kb; several discrete bands are visible up to 160 kb. No hybridization signals were obtained in the low molecular weight region (below 20 kb) using conventional agarose gel electrophoresis (data not shown).

Tobacco chromosomes contain long blocks of subtelomeric repeats

The cloned members of the HRS60 family have previously been shown to detect telomere-proximal regions of most tobacco chromosomes (Bezděk et al. 1991; Kenton

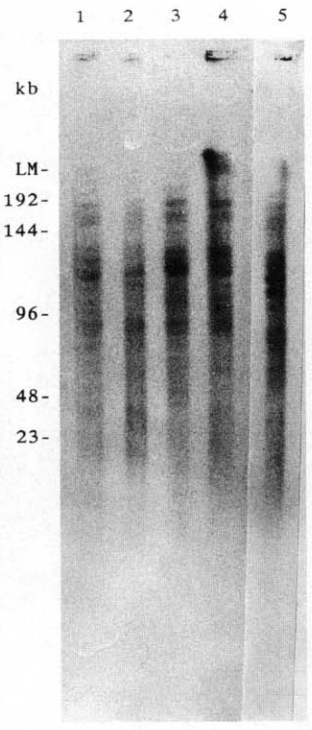

Fig. 1 Pulsed-field gel electrophoresis (PFGE) analysis of *Nicotiana tabacum* telomeres. Agarose-embedded tobacco leaf DNA was digested with *Taq*I (lane 1), *Hin*fI (lane 2), *Bst*NI (lane 3), *Alu*I (lane 4), or *Hae*III (lane 5) and fractionated on a 1% agarose gel using a CHEF-type electrophoretic apparatus [conditions, 0.1×TBE (1×TBE is 90 mM TRIS-borate, 2 mM EDTA pH 8.0) 16°C, 180 V, 24 h; switch intervals ramped from 1–12 s]. The separated DNA was transferred to Hybond N+ membrane and hybridized to a digoxygenin (DIG)-labeled $(TTTAGGG)_n$ DNA probe. Size markers derived from concatemers of phage lambda DNA digested with *Hin*dIII are indicated. (*LM* limit mobility)

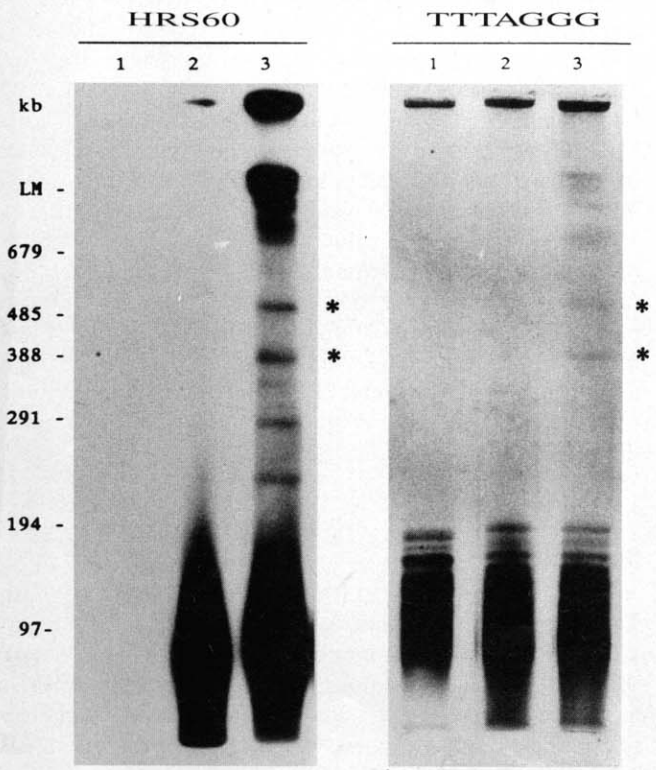

Fig. 2 PFGE analysis of *N. tabacum* subtelomeric repeats. Agarose-embedded tobacco leaf DNA was digested with *Hae*III (lanes 1), *Alu*I (lanes 2), or *Hin*dIII (lanes 3) and subjected to PFGE (0.5×TBE, 16°C, 190 V, 24 h; switch intervals ramped from 10–60 s). The membrane was hybridized to the ^{32}P-labeled HRS60 probe, exposed to film and then rehybridized to the DIG-labeled $(TTTAGGG)_n$ DNA probe. The positions of fragments hybridizing with both probes are marked with *asterisks*. Size markers derived from concatemers of lambda DNA are indicated

et al. 1993). To examine the long-range physical structure of these arrays we performed restriction enzyme digestions, followed by separation in PFGE, blotting and subsequent hybridization with the HRS60 and telomeric probes (Fig. 2). When frequently cutting enzymes were used (*Taq*I, *Hae*III, *Hin*fI, *Mbo*I, *Bst*NI), the hybridization signal of HRS60 sequences is distributed in a low molecular weight region (180 bp-1 kb) as a 180 bp ladder on a conventional electrophoresis gel (Fajkus et al 1992). Correspondingly, no hybridizing fragments can be detected on a blot of, e.g., *Hae*III-digested DNA resolved by PFGE under conditions suitable for separation of fragments in the range of 50–800 kb. However, when DNA was digested with *Alu*I, most of the HRS60-homologous sequences migrated as an unresolved high molecular weight fraction with an average size of 50–200 kb. Under these conditions bulk DNA was cut with *Alu*I into shorter fragments of less than 5 kb. These results suggest that the subtelomeric sequences are arranged in large blocks of monotonously repeated basic motifs of 180 bp without any longer intervening sequences. When DNA was cut with *Hin*dIII, most fragments hybridizing to the HRS60 probe produced a signal smear in the range of 50–200 kb. Two of the minor larger fragments co-hybridized with the telomeric probe, suggesting a close linkage of HRS60 and telomeric clusters on some chromosomes (the average fragment size of tobacco DNA cut with *Hin*dIII is approximately 8 kb, unpublished observation).

Chromatin structure of tobacco telomeric and subtelomeric repeats

In our previous report (Fajkus et al. 1992) we studied the structure of tandemly arranged repetitive sequences in tobacco chromatin and found precise positioning of nucleosomes on the subtelomeric HRS60 sequences. We wished to determine whether the telomeric chromatin of tobacco DNA is organized in a nucleosomal structure similar to that of subtelomeric chromatin comprising HRS60 sequences. To examine the nucleosomal structure, isolated nuclei were digested with MNase for increasing times and the purified DNAs were separated on agarose gels (Fig. 3). Hybridization with the HRS60 probe revealed a periodic structure of the subtelomeric chromatin, with clearly distinguishable bands corresponding to the oligomeric subunits; the subunit structure of $(TTTAGGG)_n$ repeats was not so pronounced on agarose gels since the bands were often smeared and heterogeneous. However, when the same DNA was analyzed on a high-resolution polyacrylamide gel the ladders of partial digestion products characteristic of nucleosomes could be detected (Fig. 4). Telomeric chromatin appeared to be slightly more resistant to MNase cleavage than the rest of the chromatin, since most telomeric DNA peaked as trimers and tetramers after the longest digestion interval – most of the bulk and subtelomeric chromatin was cut into mono- and dinucleosomes (Fig. 4, lane 5). Telomeric particles migrated

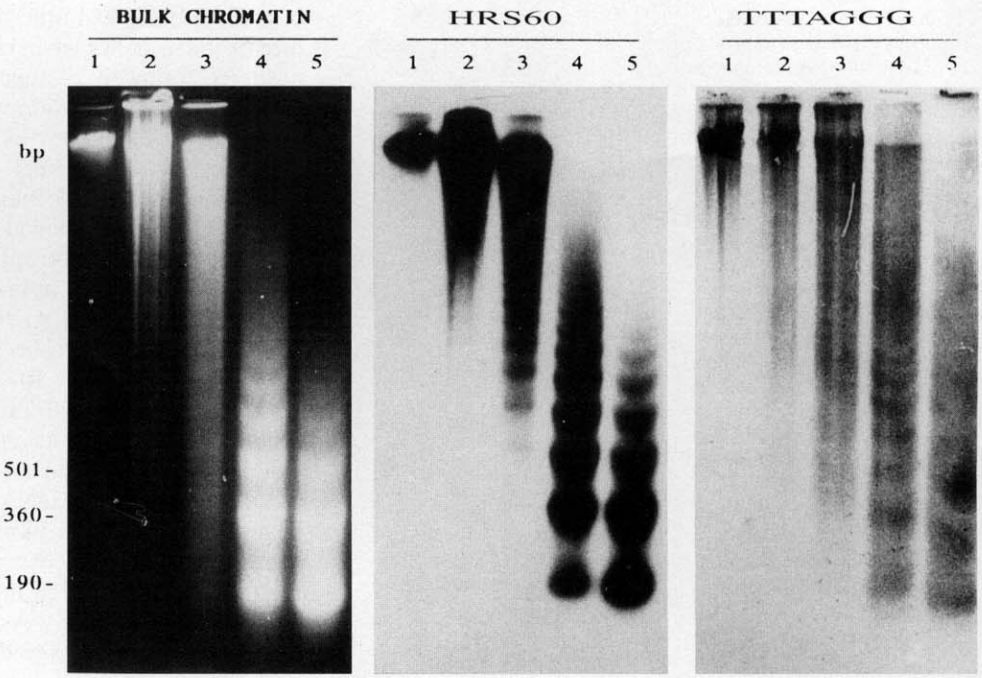

Fig. 3 Nucleosome ladders generated by micrococcal nuclease (MNase) digestion of tobacco leaf nuclei. The nuclei were digested for 0 (lanes 1), 1 (lanes 2), 3 (lanes 3), 10 (lanes 4), or 30 (lanes 5) min with 0.1 U of MNase/μg DNA at 37°C. The purified DNAs were separated through a 1.5% agarose gel, transferred to Hybond N+ membrane and subsequently hybridized with HRS60 and $(TTTAGGG)_n$ DNA probes in the same order as in Fig. 2. *Left panel* fluorograph of ethidium bromide stained gel, before Southern transfer, showing the distribution of bulk cellular DNA. *Msp*I-digested pUC19 DNA was used to provide size markers

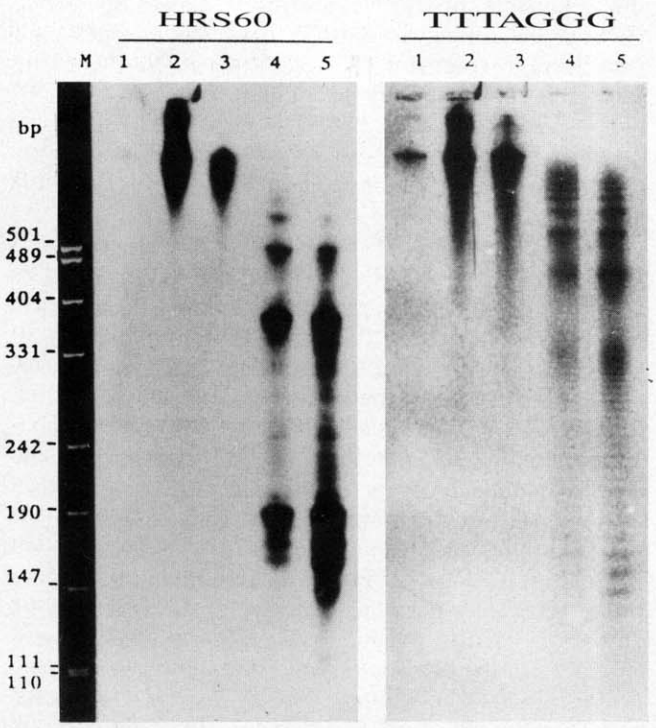

Fig. 4 Fractionation of tobacco chromatin by high resolution denaturating polyacrylamide gel electrophoresis. The DNA samples were the same as in the experiment shown in Fig. **3**. Nuclei were digested with MNase for 1, 3, 10, or 30 min (lanes 2–5). Control DNA from undigested nuclei is shown in lanes 1. The hybridizations were performed on the same membrane as described in Fig. 3. Lane M contained ethidium bromide stained pUC19 *Msp*I fragments as size markers

faster than the corresponding particles of the subtelomeric HRS60 chromatin. Molecular weights of telomeric and subtelomeric particles were determined after the 10 min digestion interval and plotted as a function of band number. Telomeric chromatin was cleaved with MNase at 157±5 bp intervals, indicating a close packing of nucleosomes. More typical ladders of HRS60 subtelomeric chromatin yielded a repeat length of 180±5 bp, which is similar to the average repeat length of tobacco leaf bulk chromatin. As shown in Fig. 4 there was no evidence for a discrete band in the position expected of the telomeric mononucleosome. Instead, a homogenous repetitious ladder of subnucleosomal fragments spaced at 7 bp intervals was visible from the bottom of the gel up to the position of the putative telomeric dinucleosome.

Discussion

The amphidiploid tobacco genome is composed of two ancestral genomes that originate from two evolutionarily relatively distant *Nicotiana* species (Goodspeed 1954). Its chromosomes have recently been characterized by molecular cytogenetics showing co-localization of telomeric $(TTTAGGG)_n$ and major subtelomeric HRS60 repeats on most chromosome ends (Kenton et al. 1993). In this study, we show that the length of tobacco telomeric $(TTTAGGG)_n$ repeats is within the range of 60–160 kb. Assuming that most are 90–130 kb long they account for approximately 0.1%–0.2% of the total genome. We further show that blocks of subtelomeric HRS60 repeats are of at least the same length as the telomeric repeat blocks (50–200 kb) and do not contain

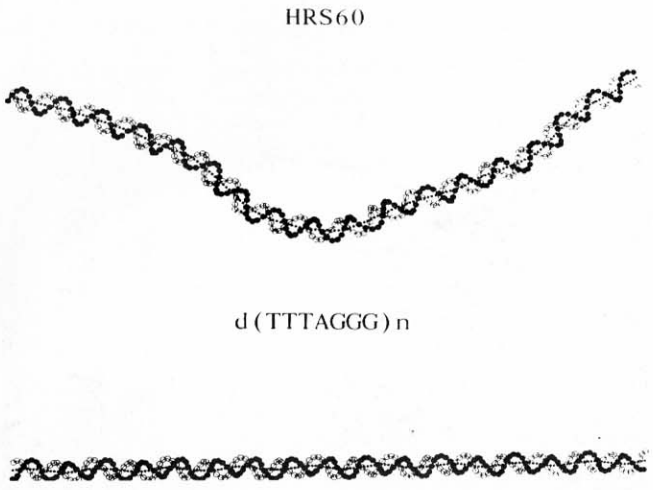

Fig. 5 In-plane projection of the DNA path plots of the subtelomeric HRS60 and the $(TTTAGGG)_n$ repeats predicted by the software CURVATURE

any longer intervening sequences. Thus, the structure of tobacco chromosome ends, consisting of two prominent domains, appears to be similar to that in tomato (Broun et al. 1992).

The chromatin structure of telomeres has been studied in several organisms. To our knowledge no attempts have been made to characterize telomere-specific chromatin in plants. We have found that in tobacco the telomeric chromatin is digested by MNase into distinct oligomeric subunits resembling regular nucleosomal particles. The cleavage sites are spaced at 157±5 bp intervals. Subtelomeric chromatin represented by HRS60 sequences possesses a nucleosome repeat length typical for bulk tobacco leaf chromatin (180±5 bp). Thus, in tobacco most telomeric and subtelomeric repeats appear to have the canonical chromatin organization. In rat and human the telomeric chromatin was shown to be organized mostly in a nucleosome-like structure with a repeat length of 150–160 bp (Makarov et al. 1993; Tommerup et al. 1994). Our estimated value of the repeat length in tobacco is 157±5 bp, suggesting that an unusually small basic subunit is probably a common feature of tightly packed telomeric chromatin both in plants and mammals.

Both plant and mammalian telomeres, however, differ from those of lower eukaryotes, in which a nonnucleosomal structure of telomeres was found (Budarf and Blackbourn 1986; Wright et al. 1992). A non-canonical structure of telomeric chromatin was also detected in some human cell lines in which telomeres were significantly shortened, suggesting the presence of nucleosome-free regions in the terminal domains (Tommerup et al. 1994). Since the majority of long telomeric repeats in tobacco seem to be packaged into canonical nucleosome arrays, short regions of non-canonically organized chromatin (if present) might escape detection by the MNase assay. Clear differences in the properties of telomeric and subtelomeric chromatin can be revealed on comparing MNase digestion profiles on high-resolution PAGE: higher order telomeric chromatin appears to be somewhat more resistant to MNase cleavage than the rest of the chromatin (Fig. 4). However, once it is cut into dimeric and monomeric particles these subunits appear to be remarkably hypersensitive to overdigestion with MNase, forming a ladder of regularly spaced fragments with a 7 bp periodicity. A similar observation was made in mammalian cells where subnucleosomal fragmentation of telomeric mononucleosomes resulted in a 6 bp ladder. In both plants and mammals the repeat length of subnucleosomal ladders correlates strikingly with the number of nucleotides in the basic repeat (7 bp repeat in plants, 6 bp in animals) suggesting the presence of a single nuclease-sensitive site. We propose that the cleavage may occur at the 5′TA3′ dinucleotide which has been shown to be preferentially attacked by MNase (Drew 1984) and which is also present in most animal and plant telomeric repeats. A precise 7 bp MNase ladder further indicates that long tobacco telomeres are composed of essentially homogeneous $(TTTAGGG)_n$ repeats and that other simple repeats are probably underrepresented (if present at all).

The fact that trimeric and multimeric telomeric particles are relatively resistant to MNase attack further suggests that the observed hypersensitivity of mono- and dimeric particles cannot be explained solely by the sequence specificity of MNase. This is supported by a recent observation of Tommerup et al. (1994) who found that the overall rate at which MNase digests naked telomeric DNA is approximately the same as that for bulk genomic sequences. We propose that the relative "instability" of telomeric mono- and dinucleosomes may be explained by loose association and/or instability of histone octamer – DNA interactions along the sequence, caused by the absence of signals for histone core positioning within the monotonous $(TTTAGGG)_n$ repeat. Such signals have been determined for other sequences and were often found to be associated with curved DNA (Travers 1987). We have analyzed the DNA curvature of both telomeric and subtelomeric sequences by computer modelling using the software CURVATURE (Shpigelman et al. 1993), which implements the nearest-neighbor wedge model of DNA curvature (Trifonov 1980). DNA path plots of subtelomeric and telomeric sequences are depicted in Fig. 5. It is obvious that while the subtelomeric sequence contains a distinct centre of curvature (preliminary gel retardation experiments confirmed the "curved" character of a 180 bp subtelomeric monomeric unit), the telomeric $(TTTAGGG)_n$ repeat appears to be straight. We speculate that the absence of intrinsic DNA curvature along the $(TTTAGGG)_n$ sequences may cause random polymerization of histone proteins, resulting in close spacing of nucleosome particles. Disruption of higher order structure, e.g., with MNase, may lead to higher mobility and sliding of telomeric nucleosomes, leaving the ends of nucleosomal DNA accessible to MNase attack. This may account for the relatively diffuse patterns of telomeric chromatin seen in Fig. 3 and the

hypersensitivity of mono- and dinucleosome particles to MNase cleavage. To confirm this hypothesis, in vitro nucleosome reconstitution experiments are planned. In conclusion, it seems that the telomeric repeats in mammals and higher plants have a very similar organization of chromatin despite differences in the sequence of the basic unit (TTAGGG in mammals, TTTAGGG in higher plants).

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