Characterization of the tomato (*Lycopersicon esculentum*) genome using in vitro and in situ DNA reassociation

Daniel G. Peterson, William R. Pearson, and Stephen M. Stack

Abstract: A detailed in vitro study of the kinetics of DNA renaturation, i.e., a C_0t analysis, can be used to determine the size of a genome, the relative proportions of single-copy and repetitive sequences, and the complexity of genome components. Despite the dual importance of tomato (*Lycopersicon esculentum*) as a model for basic plant research and as a crop plant, to the best of our knowledge a C_0t analysis has never been published for this species. This is probably due to difficulties associated with isolating sufficient quantities of polyphenol-free nuclear DNA from tomato. Recently we developed a technique for isolating milligram quantities of purified DNA from tomato nuclei, and we used DNA isolated in this manner to prepare a C_0t curve for the tomato genome. Analysis of the C_0t data indicates that the tomato genome (1C) consists of approximately 0.86 pg of DNA. In agreement with earlier molecular studies, the C_0t analysis suggests that most (~73%) of the tomato genome is composed of single-copy sequences. Since 77% of the DNA in tomato chromosomes is found in constitutive heterochromatin, many of the single-copy sequences must reside in heterochromatin, an unexpected arrangement, considering that the constitutive heterochromatin of most species is predominantly repetitive DNA. To determine the distribution of repetitive and single-copy DNA along tomato pachytene chromosomes, we used hydroxyapatite-purified C_0t fractions as probes for fluorescence in situ hybridization (FISH). Our FISH results indicate that highly repetitive DNA hybridizes almost exclusively with heterochromatin. While single-copy DNA comprises most of the DNA in euchromatin, heterochromatin contains the majority of single-copy DNA sequences, an observation consistent with our C_0t data and previous cytological studies.

Key words: tomato, *Lycopersicon esculentum*, genome size, heterochromatin, euchromatin, DNA reassociation, fluorescence in situ hybridization, FISH, C_0t.

Résumé : Une étude détaillée de la cinétique de la renaturation de l'ADN in vitro, c'est-à-dire une analyse C_0t, peut être employée pour déterminer la taille d'un génome, les proportions relatives d'ADN simple copie et d'ADN répété de même que la complexité des composantes du génome. Malgré l'importance de la tomate (*Lycopersicon esculentum*) d'abord en tant qu'espèce modèle en recherche fondamentale et ensuite en tant qu'espèce cultivée importante, au meilleur de la connaissance des auteurs, aucune analyse C_0t n'a été publiée pour cette espèce. Cette lacune est vraisemblablement due aux difficultés qui sont rencontrées dans l'extraction, en quantités suffisantes, d'ADN génomique nucléaire exempt de polyphénols chez la tomate. Récemment, les auteurs ont mis au point une technique qui permet d'isoler des milligrammes d'ADN purifié à partir de noyaux de tomate. De l'ADN isolé par cette technique a été employé afin de préparer une courbe C_0t pour le génome de la tomate. L'analyse des données C_0t indique que le génome (1C) de la tomate comprend environ 0,86 pg d'ADN. En accord avec les études moléculaires précédentes, l'analyse C_0t suggère que la majeure partie (~73%) du génome de la tomate est composé de séquences à simple copie. Puisque ~77% de l'ADN des chromosomes de la tomate se trouve dans des régions d'hétérochromatine constitutive, plusieurs des séquences d'ADN à simple copie doivent être localisées parmi l'hétérochromatine, une situation inattendue si on considère que l'hétérochromatine constitutive est composée surtout d'ADN répété chez la plupart des espèces. Afin de déterminer la distribution des séquences répétées et à simple copie sur les chromosomes en pachytène, les auteurs ont employé des fractions C_0t purifiées sur hydroxyapatite en tant que sondes pour réaliser des hybridations in situ à fluorescence. Les résultats de ces hybridations indiquent que l'ADN très répétitif hybride presque exclusivement avec l'hétérochromatine. Bien que l'euchromatine est formée presque entièrement d'ADN à simple copie, la majeure partie de l'ADN à simple copie se trouve dans l'hétérochromatine. Cette observation concorde avec les résultats de l'analyse C_0t et avec les études cytologiques antérieures.

Mots clés : tomate, *Lycopersicon esculentum*, taille du génome, hétérochromatine, euchromatine, réassociation d'ADN, hybridation in situ à fluorescence, FISH, C_0t.

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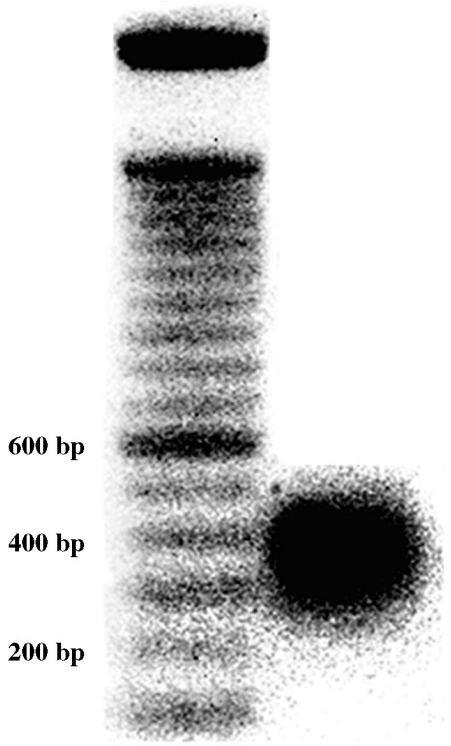
Introduction

Tomato (*Lycopersicon esculentum*) is an economically important crop plant that has proven to be a useful model in the study of plant genetics, molecular biology, and chromosome structure (for review see Rick 1991). All tomato chromosomes possess large blocks of pericentric constitutive heterochromatin (Ramanna and Prakken 1967). Using Feulgen densitometry we determined that 77% of the tomato genome is contained within these blocks of heterochromatin (Peterson et al. 1996). In most species, repetitive DNA sequences appear to make up the bulk of constitutive heterochromatin (e.g., Appels et al. 1978, 1981; Deumling and Greilhuber 1982), and thus one might predict that the tomato genome contains a considerable amount of repetitive DNA. However, based upon analysis of a sheared tomato genomic library, S.D. Tanksley and his colleagues have suggested that the tomato genome is composed of >70% single-copy DNA sequences (Zamir and Tanksley 1988; Ganal et al. 1988).

To make an independent determination of the amount of single-copy DNA in the tomato genome, we examined the reassociation of tomato DNA in vitro by performing a C_0t analysis[2] (for reviews see Britten et al. 1974; Hood et al. 1975; and Britten and Davidson 1985). Based upon our C_0t data, tomato possesses a 1C DNA content of 0.86 pg, and in agreement with the aforementioned molecular studies, most (~73%) of the tomato genome is composed of single-copy sequences. Consequently, it would appear that tomato heterochromatin must contain a significant amount of single-copy DNA. To test this idea, purified highly repetitive, middle-repetitive, and single-copy DNA fractions were used as probes for fluorescence in situ hybridization (FISH) to pachytene chromosomes. Our FISH analysis demonstrates that tomato heterochromatin contains substantial quantities of single-copy and middle-repetitive DNA, while tomato euchromatin contains little if any highly repetitive sequences.

[2] Briefly, in C_0t analysis, samples of sheared nuclear DNA in sodium phosphate buffer are denatured and allowed to reassociate for various periods of time. The product of a sample's nucleotide concentration (moles of nucleotides per litre), its reassociation time in seconds, and an appropriate buffer factor based upon cation concentration gives the sample's C_0t value (see Britten et al. 1974). Once a sample has reassociated to a desired C_0t value, the sample is run onto a hydroxyapatite column. The single-stranded DNA and the double-stranded DNA (reassociated sequences) are eluted separately by manipulating the buffer concentration in the column. The logarithm of a C_0t value is graphed against the corresponding fraction of the genome that had remained single-stranded to yield a C_0t point. A graph of C_0t points ranging from little or no reassociation until reassociation approaches completion is called a C_0t curve. Because a DNA sequence reassociates at a rate that is directly proportional to the number of times it occurs in the genome, sequences that occur more than once in a genome (repetitive DNA) reassociate at lower C_0t values than sequences that occur only once per genome (single-copy DNA). From an analysis of a C_0t curve, one can determine genome size, relative proportions of single-copy and repetitive sequences, the fraction of the genome occupied by each frequency component, and the complexity of the sequences in each frequency component.

Fig. 1. Physical shearing of tomato DNA. The image has been reversed so fluorescent bands appear dark on a light background. In the left lane of the gel is a 100-bp ladder (Gibco BRL). The distance between each lower band of the ladder is equivalent to 100 bp. In the right lane of the gel is tomato nuclear DNA that has been sheared using a Vertis homogenizer. Fragment lengths range from 275 to 500 bp, with a mean fragment length of 375 bp.



Materials and methods

Buffer preparation

Dibasic sodium phosphate (Na_2HPO_4) and monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$) were mixed with deionized water to yield solutions of approximately 0.5 M. The actual molarities of the solutions were determined using an American Optical Abbe refractometer and a table of refractive indices obtained from Leica (Buffalo, N.Y.). Each solution was diluted with deionized water until its refractive index indicated a concentration of 0.48 M. Equal parts of 0.48 M monobasic sodium phosphate and 0.48 M dibasic sodium phosphate were mixed to produce a 0.48 M sodium phosphate buffer (0.48 M SPB). For denaturation–renaturation studies, the stock solution was used at full strength or diluted with deionized water to produce 0.12 and 0.03 M SPB.

Isolation and preparation of DNA for C_0t analysis

Tomato nuclear DNA was isolated from tomato seedlings (var. Cal-Ace) according to Peterson et al. (1997) and Peterson and Stack (1997). This DNA had an A_{260}/A_{280} ratio (adjusted for light scatter at 320 nm) of >1.8. Two to four milligrams of the DNA was dissolved in a small volume (<2 mL) of TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 7.0) and mixed with 10 mL of ultrapure glycerol (USB). DNA was sheared using a Vertis homogenizer, as described by Davidson et al. (1973). Blade velocity and homogenization time were adjusted so that fragments with a mean length of 375 bp were produced (range = 275–500 bp), as determined by agarose-gel electrophoresis (Fig. 1). To remove glycerol, the mixture was dialyzed into TE buffer for 24 h. The resulting DNA solution was run through a

Chelex (Bio-Rad) column to remove metal ions (Britten et al. 1974). The eluant was mixed with 1/20 volume of 3 M sodium acetate (pH 5.2) and two volumes of 100% ethanol and spun for 1 h at 32 000 rpm at 4°C in a Beckman L755 ultracentrifuge using a SW41 rotor. Supernatants were decanted, and the DNA pellets were dried overnight. DNA pellets were redissolved in 0.03, 0.12, and 0.48 M SPB. DNA solutions were spun briefly in a microcentrifuge to pellet any particulates, and supernatants were transferred to new siliconized microcentrifuge tubes.

DNA melting curves

Melting curves for sheared tomato DNA in 0.03 and 0.12 M SPB were generated using a Beckman DU650 spectrophotometer equipped with a multicell gasket-sealed cuvette holder heated by a Peltier temperature controller. Both DNA samples and appropriate blanks (0.03 and 0.12 M SPB containing no DNA) were degassed, loaded into cuvettes, and placed in the cuvette holder. The temperature of the cuvettes was slowly increased from 25 to 110°C. At each 1°C increase in temperature, cuvettes were allowed to stabilize for 1 min before A_{260} readings were taken. To maintain baseline linearity, at each degree, the A_{260} of the blank was automatically subtracted from the A_{260} of its corresponding DNA sample. As the DNA was heated, melting curves were generated for both DNA samples. After denaturation was complete, melting temperatures (T_ms) were determined by the spectrophotometer software using first-derivative analysis. The T_m of sheared tomato DNA in 0.48 M SPB was not determined by spectrophotometry, but rather was estimated on the basis of the T_ms of the 0.03 and 0.12 M SPB samples (see Results and discussion).

Preparation of C_0t points

All subsequent UV spectrophotometry was performed using a Hewlett Packard (HP) 8453 spectrophotometer connected to a HP Vectra VL Series 3 computer. Spectrophotometric readings were stored and analyzed using the HP computer program UV-Visible ChemStation. The program was set so that A_{260} and A_{280} readings were automatically adjusted for light scatter at A_{320}. A DNA concentration curve was prepared using dilutions of a DNA solution of known concentration (10 mg/mL herring sperm DNA; Gibco BRL). The linear equation of the calibration curve was entered into the computer, so that when any sample was examined, the DNA concentration was determined automatically. Tomato DNA solutions used to prepare C_0t points varied in concentration from 3 to 16 624 μg/mL. All DNA solutions had an adjusted A_{260}/A_{280} ratio of >1.8. DNA samples were sealed in siliconized glass ampoules or siliconized glass microcapillary tubes. Each ampoule or capillary tube contained approximately 100 μg of DNA, regardless of buffer molarity or sample volume. Ampoules and capillary tubes containing DNA were frozen in liquid nitrogen and stored at –70°C for later use.

Hydroxyapatite (HAP) was prepared according to Bernardi (1971). A Bio-Rad water-jacketed column (cat. No. 737-6131) fitted with a Bio-Rad flow adapter (cat. No. 738-0015) was used for HAP chromatography. This column was maintained at 60°C using a MGW Lauda circulating water bath. The tube from which eluant exited the column was directly connected to a flow-cell cuvette in the spectrophotometer. The tube exiting the flow cell was connected to an ISCO Wiz™ peristaltic pump set at a speed of 0.75 mL/min. A layer of glass beads 3 mm deep was placed on top of the fret at the top of the flow adapter. A 1-cm^3 layer of hydroxyapatite was added. Five millilitres of 0.48 M SPB (60°C), 5 mL of 0.12 M SPB (60°C), and 10 mL of 0.03 M SPB (60°C) were run through the HAP column prior to loading a DNA sample. The column was never allowed to run dry and was agitated periodically to prevent formation of channels through the HAP (Britten et al. 1974). A fresh HAP column was prepared for each DNA sample.

To prepare a C_0t point, an ampoule or capillary tube was thawed and placed in boiling water for 5–10 min. The sample was then immediately placed in a water bath set at a temperature 25°C below the T_m for sheared tomato genomic DNA in the same buffer as the sample. Each sample was incubated to a desired C_0t value, as described by Britten et al. (1974). At the end of the incubation period, samples in 0.48 or 0.12 M SPB were immediately diluted in a 100-fold excess of 0.03 M SPB (60°C) and loaded onto a HAP column. Samples in 0.03 M SPB were not diluted, but rather were applied directly to a HAP column and quickly pulled into the column by temporarily speeding up the peristaltic pump. Based on A_{260} readings of the eluant, no DNA came through the column during loading. Once a sample had almost completely entered the HAP column, 0.12 M SPB was added to elute single-stranded DNA. Elution of single-stranded DNA (ssDNA) was visualized on the computer screen as a peak in A_{260}. Eluant containing ssDNA was collected in a graduated polypropylene tube. After the ssDNA had been collected, 0.48 M SPB was added to the column to elute double-stranded DNA (dsDNA). The dsDNA also eluted in a single peak and was collected in a separate tube. The volumes of the ssDNA and dsDNA eluants were determined. One millilitre of each eluant was placed in its own siliconized microcentrifuge tube and spun at 4000 × *g* to pellet any particulates that might interfere with absorbance. A 0.9-mL aliquot of the centrifuged ssDNA eluant was mixed with 0.1 mL of aqueous 10 N KOH to denature any DNA duplexes. A solution consisting of 9 parts 0.12 M SPB and 1 part 10 N KOH was used to blank the spectrophotometer. The A_{260} value (adjusted for light scatter at 320 nm) of the ssDNA–KOH mixture was determined. Similarly, a 0.9-mL aliquot of the centrifuged dsDNA eluant was mixed with 0.1 mL of aqueous 10 N KOH to denature duplexes. The spectrophotometer was then blanked with a solution composed of 9 parts 0.48 M SPB and 1 part 10 N KOH, and the adjusted A_{260} of the denatured dsDNA–KOH mixture was determined. For a particular C_0t value, the percentage of ssDNA (percent ssDNA) was calculated as follows:

$$[1] \quad \frac{(V_{ss} \times A_{ss}) \times 100}{(V_{ss} \times A_{ss}) + (V_{ds} \times A_{ds})} = \%\ \text{ssDNA}$$

where

V_{ss} = total volume of the single-strand fraction

V_{ds} = total volume of the double-strand fraction

A_{ss} = A_{260} (adjusted for light scatter) for the KOH-denatured single-strand fraction

A_{ds} = A_{260} (adjusted for light scatter) for the KOH-denatured double-strand fraction

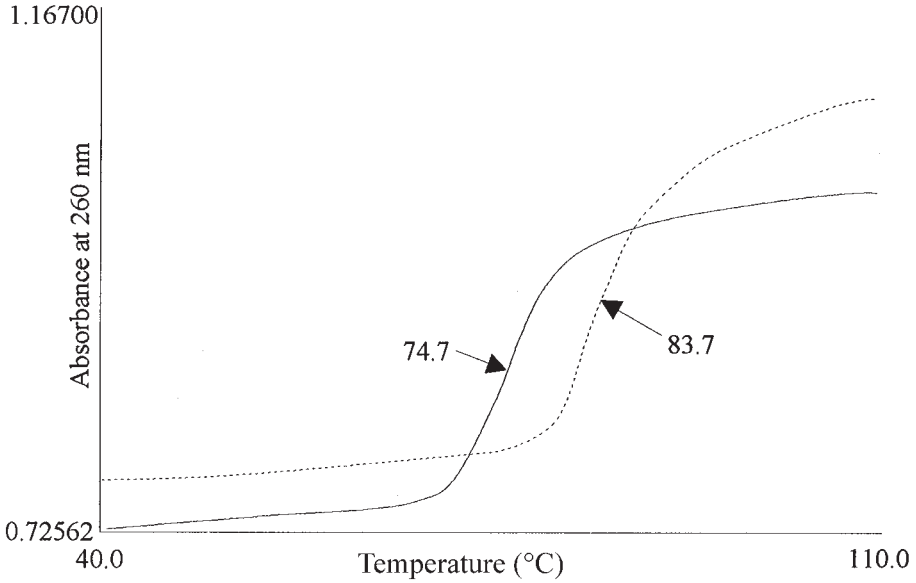
The logarithms of C_0t values were plotted against corresponding percent ssDNA values. A least-squares analysis of the C_0t data was performed using the computer program of Pearson et al. (1977), and a best-fit curve was generated for the data points.

FISH to tomato pachytene chromosomes

Pachytene microsporocytes were removed from tomato anthers (var. Cherry), fixed in 1:3 acetic acid – ethanol, digested with enzymes to remove cell walls (1% (w/v) Onozuka R10 cellulase, 1% (w/v) Sigma desalted sulfatase, plus 1% (w/v) Sigma desalted pectinase in 1 mL of deionized water to which 1 drop of 0.1 N HCl had been added), and resuspended in 45% acetic acid, as described by Peterson et al. (1996). Two drops of microsporocyte suspension were dropped from a Pasteur pipet at a height of 10 cm onto a microscope slide covered with absolute ethanol. The ethanol was ignited and allowed to burn away.

Based upon the results of the C_0t analysis, single-copy, middle-repetitive, and highly repetitive DNA fractions were isolated using HAP chromatography as described above. The three C_0t fractions were each dialyzed into TE buffer (pH 8.0), precipitated with ethanol, and redissolved in 200 μL of TE buffer. Four micrograms of each C_0t fraction was labeled with biotin–dCTP by random priming, using Pharmacia Ready-To-Go™ DNA Labelling Beads. As a control, the

Fig. 2. Melting curves for sheared tomato genomic DNA in 0.03 M SPB (solid line) and 0.12 M SPB (dashed line). For each curve, melting temperature (i.e., 50% hyperchromicity) is indicated by an arrow.



162 bp tomato tandem repeat sequence TGRI (see Ganal et al. 1988 and Lapitan et al. 1989) was also prepared for use as a probe, using PCR (Rashtchian and Mackey 1992).

FISH was performed using a modified version of the protocol of Rayburn and Gill (1985). Microscope slides containing chromosome preparations were incubated in 2× SSC (aqueous 0.3 M NaCl plus 0.05 M sodium citrate, pH 7.0) containing 100 µg/mL "DNase-free" RNase A at 37°C for 20 min, washed in two changes of 2× SSC (37°C, 10 min each wash), rapidly dehydrated in an ethanol series at –20°C, and air-dried. Slides then were placed in 70% formamide in 2× SSC at 70°C for 2.5 min, dehydrated in an ethanol series, and air-dried. Approximately 2 µg of biotin-labeled probe (100 µL) was added to a hybridization mixture consisting of 75 µL formamide, 30 µL 50% aqueous potassium dextran sulfate, 15 µL 20× SSC (aqueous 3 M NaCl plus 0.5 M sodium citrate, pH 7.0), and 7.5 µL 10 mg/mL sheared herring sperm DNA (Gibco BRL). Hybridization mixtures containing probe DNA were heated at 97°C for 10 min and then immediately placed on ice. Fifty microlitres of hybridization mixture was placed on a slide and a siliconized cover glass (22 × 50 mm) was added. Slides were placed in sealed humid chambers and incubated at 37°C overnight. Cover glasses were washed from slides with 2× SSC (37°C). Slides were washed three times in 2× SSC, twice in 2× SSC containing 2% Tween-20 (37°C, 10 min each wash), and incubated for 20 min at 37°C in blocking buffer (0.1 M maleic acid, 0.15 M NaCl, plus 1% Boehringer Mannheim blocking reagent, pH 7.5). Both a direct and an indirect method were used to detect probe hybridization. In the direct method, slides were incubated at 37°C for 1 h in blocking buffer containing 5 µg/mL streptavidin – fluorescein isothiocyanate (FITC) conjugate (Gibco BRL). In the indirect method, slides were incubated for 1 h in blocking buffer containing 1 µg/mL mouse anti-biotin (Boehringer Mannheim), washed three times in 2× SSC containing 2% Tween-20 (37°C, 10 min each wash), and incubated for 1 h at 37°C in blocking buffer containing 3 µg/mL goat anti-mouse–FITC (Boehringer Mannheim). All slides were washed three times in 2× SSC at room temperature (10 min each wash), counterstained with 4′,6-diamidinophenylindole (DAPI; 0.5 µg/mL in aqueous 18 mM citric acid plus 165 mM Na_2HPO_4, pH 7.0) for 10 min, rinsed with deionized water, and air-dried for 10 min. Cover glasses (22 × 50 mm) were mounted using freshly prepared antifade medium (50 mM Tris, 50% glycerol, plus 1 mg/mL phenylenediamine, pH 9.0) and sealed using fingernail polish. Fluorescence microscopy was performed using an Olympus Provis AX70 microscope equipped with a narrow green filter for FITC and a wide UV filter for DAPI.

Results and discussion

T_ms and GC content of tomato DNA

Melting curves for sheared tomato DNA in 0.03 and 0.12 M SPB were generated (Fig. 2) and respective T_ms were determined using first-derivative analysis. The T_ms for tomato DNA in 0.03 and 0.12 M SPB were 74.7 and 83.7°C, respectively.

The GC content of tomato nuclear DNA was calculated using the formula of Mandel and Marmur (1968). For buffers with monovalent cation concentrations between 0.01 and 0.2 M,

[2] $$\%GC = 2.44\ (T_m - 81.5 - 16.6 \log M)$$

where

M = the molar concentration of the cation

Based on the tomato DNA sample in 0.03 M SPB (Na^+ = 0.45 M), %GC = 2.44 (74.7 – 81.5 – 16.6 log 0.045) = 38.0. If the data for tomato DNA in 0.12 M SPB (Na^+ concentration = 0.18 M) is used, %GC = 2.44 (83.7 – 81.5 – 16.6 log 0.18) = 35.5. The average of these two %GC values is 36.8.

GC content also can be calculated from the buoyant density of DNA in CsCl. According to Ingle et al. (1973), tomato main band DNA has a buoyant density in CsCl of 1.694 g/mL. Using the formula of Mandel et al. (1968):

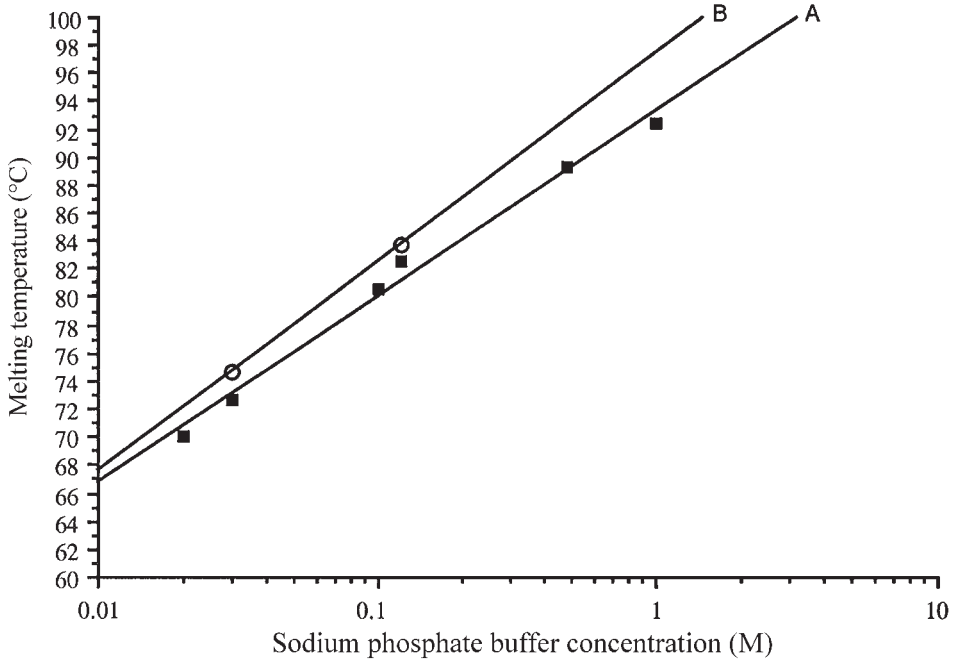
[3] $$\%GC = [(p - 1.660\ g/mL) \div 0.098] \times 100$$

where

p = observed buoyant density

Then, %GC = [(1.694 g/mL – 1.660 g/mL) ÷ 0.098] × 100 = 34.6. This value is close to the 36.8% GC we calculated from our tomato DNA melting curves.

Fig. 3. Relationship between melting temperature and SPB concentration. Using data from Stack and Comings (1979), we graphed T_m versus SPB concentration for *A. cepa* genomic DNA (line A). Each data point is represented by a black square. Analysis of the graph indicates a logarithmic correlation between these two variables ($y = 93.402 + 13.341 \log x$, $r^2 = 0.991$). Assuming that the relationship between SPB and T_m is logarithmic for other species, we fit the two T_m/SPB data points (open circles) for tomato with a logarithmic curve (line B). The equation for this line is $y = 97.465 + 14.949 \log x$. Using this equation, the T_m for tomato DNA in 0.48 M SPB is approximately (97.465 + 14.949 log 0.48 =) 92.7°C.



T_ms and reassociation temperatures

In C_0t analysis, the standard temperature at which a sample in SPB is allowed to renature is 25°C below the T_m for genomic DNA in that buffer (Britten et al. 1974). Based upon melting curves, the T_ms of tomato DNA in 0.03 and 0.12 M SPB were 74.7 and 83.7°C, and thus the renaturation temperatures used for samples in these buffers were (75 – 25 =) 50°C and (84 – 25 =) 59°C, respectively. Unfortunately, our access to the Beckman spectrophotometer was limited, and thus we did not have the opportunity to generate a melting curve for tomato DNA in 0.48 M SPB. However, based upon melting-curve data for *Allium cepa* (Stack and Comings 1979), the relationship between T_m and SPB concentration appears to be logarithmic in nature ($r^2 = 0.991$) (Fig. 3). Assuming that a logarithmic relationship between T_m and SPB concentration exists for the DNA of other species, we fitted a logarithmic curve through the T_m data points for tomato (Fig. 3). The equation of the resulting curve is

[4] $y = 97.465 + 14.949 \log x$

where

x is the molar concentration of the phosphate buffer

Thus for 0.48 M buffer, the predicted T_m for sheared tomato DNA is (97.465 + 14.949 log 0.48 =) 92.7°C. Consequently, the renaturation temperature used for tomato DNA samples in 0.48 SPB would be (93 – 25 =) 68°C. The C_0t points generated from DNA dissolved in sodium phosphate buffer of all three concentrations fall in a predictable relationship to one another (see Table 1 and Fig. 4), and thus it appears that the renaturation temperature used for samples in 0.48 M SPB was appropriate. However, even if the renaturation temperature we calculated for tomato DNA in 0.48 M SPB is slightly off, there is little difference in reassociation rates ± 10°C from the standard renaturation temperature for a particular buffer (see Fig. 1 in Britten et al. 1974).

Analysis of the C_0t data

C_0t data was analyzed using the computer program of Pearson et al. (1977). This and similar programs have been the standard means by which C_0t data have been analyzed for >20 years (e.g., Britten et al. 1974; Smith et al. 1975; Kiper and Herzfeld 1978; Stack and Comings 1979; Green et al. 1982; Leutwiler et al. 1984). The analysis providing the lowest RMS (root mean square deviation) and goodness of fit values (0.0319 and 0.0352, respectively) was a two-component fit with no other constrained variables. The results of this analysis are shown in Table 2, while the corresponding C_0t curve is shown in Fig. 4.

In all C_0t analyses, a certain fraction of the DNA has formed duplexes, even at C_0t values approaching zero. It is generally assumed that such early renaturation is due to base pairing between complementary sequences on the same DNA molecule. Such "fold-back" DNA usually accounts for 5–15% of total reassociation (e.g., Kiper and Herzfeld 1978; Stack and Comings 1979; Leutwiler et al. 1984). As shown in Fig. 4, approximately 12% of the tomato DNA had reassociated by the earliest C_0t point (10^{-6} M·s) and consequently was not included in the curve. No detectable reassociation was observed until a C_0t value of about 0.02 M·s.

Additionally, 11% of the tomato DNA did not reassociate by the highest C_0t value (20 233 M·s) and consequently was

Fig. 4. Complete C_0t curve for tomato genomic DNA and individual C_0t profiles for components 1 and 2. To generate the complete C_0t curve, a least-squares curve was fitted through the data points (open circles), using the computer program of Pearson et al. (1977). On the complete C_0t curve, the $C_0t_{1/2}$ values for components 1 and 2 are labeled. Additionally, the predicted individual renaturation profiles of isolated curve components are shown.

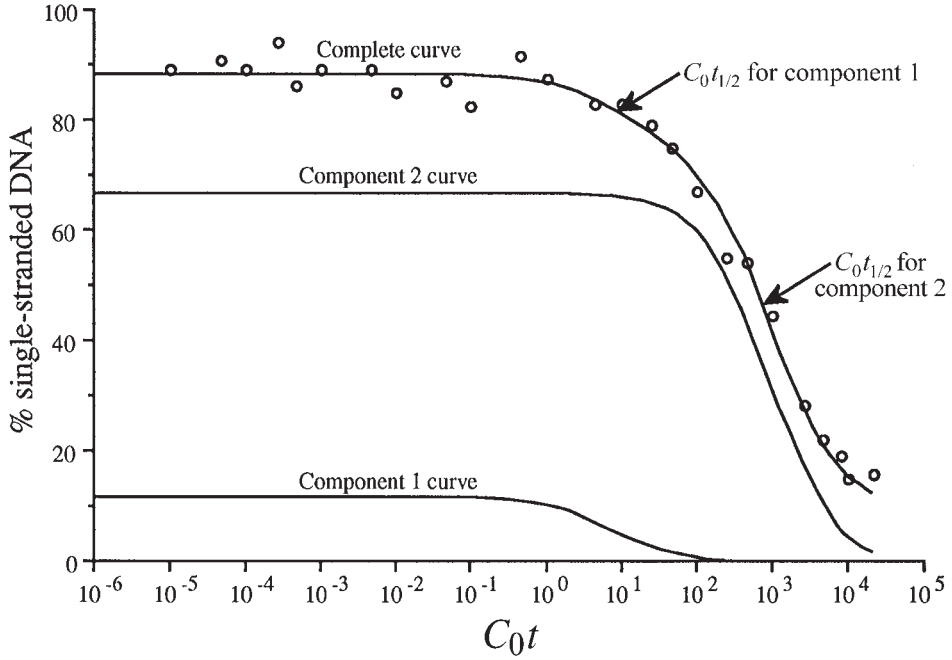


Table 1. C_0t values with corresponding percentages of single-stranded (ss)DNA.

C_0t	% ssDNA	SPB[a]
0.000 01	89.3	0.03
0.000 05	90.7	0.03
0.000 1	89.2	0.03
0.000 25	94.0	0.03
0.000 5	86.3	0.03
0.001	89.0	0.03
0.005	89.3	0.03
0.01	85.0	0.03
0.05	87.0	0.03
0.1	82.5	0.03
0.5	91.6	0.12
1	87.4	0.12
5	82.9	0.48
10	83.8	0.48
10	85.1	0.48
10	82.9	0.48
25	79.2	0.48
50	75.1	0.48
100	67.2	0.48
250	54.8	0.48
500	54.1	0.48
1 000	44.6	0.48
2 500	28.3	0.48
5 000	22.0	0.48
7 500	19.3	0.48
10 000	14.8	0.48
20 000	5.8	0.48
20 233	15.8	0.48

[a]Concentration of sodium phosphate buffer (M) in which DNA renaturation occurred.

Table 2. Least-squares analysis of tomato C_0t data using the computer program of Pearson et al. 1997.

Component	$F_{(actual)}$[a]	$F_{(adjusted)}$[b]	Complexity[c]	k[d]	$C_0t_{½}$[e]
1	0.12	0.13	1.11×10^8	0.112 300	8.90
2	0.65	0.73	5.41×10^{10}	0.001 221	819.00

[a]The fraction of the ordinate axis of the C_0t curve occupied by a particular component, i.e., the proportion of the genome found in a particular component.

[b]The $F_{(actual)}$ of each component divided by 1 minus the fraction of the genome in unannealable (damaged) DNA (i.e., 1 – 0.11 = 0.89). The $F_{(adjusted)}$ value for a component is roughly the fraction of the genome that would be occupied by that component if one assumes that unannealable DNA is similar in sequence and proportion to the DNA in components 1 and 2.

[c]The length in nucleotide pairs of the longest nonrepeating sequence.

[d]The observed reassociation rate for a component ($M^{-1} \cdot s^{-1}$) determined from the complete C_0t curve.

[e]C_0t value (M·s) on the abscissa of the complete C_0t curve at which half the DNA of a component has reassociated.

not included in the curve. DNA that does not reassociate by such a high C_0t value will most likely never reassociate. This "unannealable" DNA is presumably composed of damaged DNA that cannot form duplexes capable of being bound by HAP (e.g., Kiper and Herzfeld 1978; Stack and Comings 1979; Leutwiler et al. 1984).

The curve itself consists of a relatively fast reassociating component (component 1) and a more slowly reassociating component (component 2) (Fig. 4). Component 1 contains approximately 12% of the genome and exhibits 50% reassociation by a C_0t of 8.9 M·s, i.e., $C_0t_{1/2}$ = 8.9. Component 2 contains approximately 65% of the genome and has a $C_0t_{1/2}$ of 819.0 M·s. Because the reassociation rate (k) for a component

is the inverse of its $C_0t_{1/2}$ (i.e., $k = 1 \div C_0t_{1/2}$), components 1 and 2 have reassociation constants of 0.112 300 and 0.001 221 $M^{-1}\cdot s^{-1}$, respectively.

Molecular evidence strongly suggests that tomato is a true diploid, as most gene sequences occur only once per 1C genome (Bernatzky and Tanksley 1986; Helentjaris et al. 1988). Consequently, it is likely that component 2 of the tomato C_0t curve is composed of single-copy DNA sequences. According to Britten and Davidson (1985), for the single-copy component of a C_0t curve,

[5] $k = R \div G$

where

R = the rate constant ($M^{-1}\cdot s^{-1}$), irrespective of complexity

G = genome size in nucleotide pairs

Under standard conditions (fragments 500 bp long, 0.12 M SPB, and assay by HAP chromatography), $R = 10^6\ M^{-1}\cdot s^{-1}$. While the conditions used in this study were not entirely standard (e.g., mean DNA fragment length = 375 bp), they were generally close enough to standard conditions that the R value of $10^6\ M^{-1}\cdot s^{-1}$ can be used. Based upon independent reports that estimate the tomato genome size to be 0.7–1 pg (Sparrow and Mischke 1961; Galbraith et al. 1983; Michaelson et al. 1991), the predicted k value of the single-copy component of the tomato genome would lie between 0.001 48 and 0.001 04 $M^{-1}\cdot s^{-1}$. Component 2 of our tomato C_0t curve has a k value of 0.0012 $M^{-1}\cdot s^{-1}$, a value that fits comfortably within the range of predicted k values for tomato single-copy sequences. This strongly supports the idea that component 2 is composed of single-copy DNA.

Assuming that component 2 is composed of single-copy sequences, component 1 must be composed of repetitive DNA. Since component 2 has a repetition frequency of 1, the repetition frequency of the DNA in component 1 can be determined by dividing the k value of component 1 by the k value of component 2 (Hood et al. 1975), as follows: 0.1123 ÷ 0.001 221 = 92. Thus, on average, each sequence in component 1 is repeated 92 times.

If one assumes that the damaged DNA that does not reanneal even at the highest C_0t values is reflective in sequence and relative proportion of the DNA in components 1 and 2, the percentage of repetitive DNA in the genome may be nearer (0.12 ÷ (1.0 – 0.11) =) 13%. Likewise, the percentage of the genome made up of single-copy sequences may be nearer (0.65 ÷ (1.0 – 0.11) =) 73%. Based on molecular biology studies, S.D. Tanksley and his colleagues have also suggested that >70% of the tomato genome is single-copy DNA (Ganal et al. 1988; Zamir and Tanksley 1988).

Genome size

The genome size of an organism is inversely proportional to the observed reassociation rate for the single-copy component of that organism (Britten and Davidson 1985). Subsequently, tomato genome size (1C) can be calculated from the reassociation constant of component 2 ($k = 0.0012\ M^{-1}\cdot s^{-1}$), using the formula

[6] $k = R \div G$

where

G = genome size in base pairs

$R = 10^6\ M^{-1}\cdot s^{-1}$

Thus, $0.0012\ M^{-1}\cdot s^{-1} = 10^6\ M^{-1}\cdot s^{-1} \div G$ and $G = 10^6\ M^{-1}\cdot s^{-1} \div 0.0012\ M^{-1}\cdot s^{-1} = 8.33 \times 10^8$ bp.

Because 1 pg of DNA contains 0.965×10^9 bp (Bennett and Smith 1976), the 1C DNA amount for tomato is ((8.33×10^8 bp) (1 pg ÷ 0.965×10^9 bp) =) 0.86 pg. This value falls within the range of genome-size estimates that have been made for tomato based upon other techniques (e.g., 1.0 pg, Sparrow and Mischke 1961; 0.70 pg, Galbraith et al. 1983; 0.95 pg, Michaelson et al. 1991).

Repetitive DNA families in tomato

Only one distinguishable repetitive DNA component was detected by C_0t analysis. However, several different repeated DNA sequences are found in the tomato genome (see Ganal et al. 1988 and Lapitan et al. 1989). Subsequently, it is likely that component 1 of our C_0t curve is actually composed of a number of different repetitive DNA sequences.

For example, the nucleolus organizer region on chromosome 2 contains approximately 2300 copies of the 9.1 kb 45S ribosomal DNA sequence. If this is true, the expected $C_0t_{1/2}$ of the rDNA sequence can be determined from the $C_0t_{1/2}$ of component 2 as follows: 819 M·s ÷ 2300 = 0.35 M·s. Because rRNA molecules possess a great deal of secondary structure (i.e., intramolecular base pairing; Noller and Woese 1981), it is possible that some of the rDNA sequences contribute to fold-back DNA.

In addition to rDNA, three other nontelomeric tomato genomic repeat sequences (TGRI, TGRII, and TGRIII) have been characterized. TGRI is a 162 bp tandemly repeated satellite sequence located near most telomeres and at a few interstitial sites. TGRI is repeated 77 000 times and accounts for 1.75% of the tomato genome. Subsequently, the expected $C_0t_{1/2}$ for the TGRI repeat is (819 M·s ÷ 77 000 =) 0.011 M·s. TGRII is an interspersed repeat sequence of unknown complexity that is found 4200 times in the tomato genome and thus has an expected $C_0t_{1/2}$ of (819 M·s ÷ 4200 =) 0.195 M·s. Similarly, TGRIII is a dispersed repeat sequence of unknown complexity that is found approximately 2100 times in the tomato genome and has an expected $C_0t_{1/2}$ of (819 M·s ÷ 2100 =) 0.390 M·s. The calculation of 92 as the mean repetition frequency of component 1, indicates that most repeated sequences in the tomato genome are present in lower copy numbers than the repeats discussed above.

Although tomato telomeres have been studied using molecular biology techniques (Ganal et al. 1991), to our knowledge the fraction of the genome occupied by the telomeric repeat sequence (TT(T/A)AGGG) has yet to be determined.

FISH to pachytene chromosomes

According to Britten and Davidson (1985), 80% of the DNA in a particular C_0t curve component will renature over two C_0t decades. Based upon our C_0t curve, the $C_0t_{1/2}$ for the single-copy DNA component is 819.0. Thus, 80% of tomato single-copy DNA should renature between a C_0t of 81.9 and one of 8190. DNA sequences renaturing at C_0t values > 819 should be

almost exclusively single-copy in nature. Thus, we isolated DNA with a $C_0t > 819.0$ for use as a single-copy FISH probe.

We categorized DNA sequences that were repeated 1000 times or more as "highly repetitive." The $C_0t_{1/2}$ of DNA sequences repeated 1000 times is (819.0 ÷ 1000 =) 0.819. Thus, for our highly repetitive DNA probe, we isolated DNA with a C_0t value ≤ 0.819. This fraction should contain almost no single-copy sequences. Some of the highly repetitive DNA fraction is undoubtedly fold-back sequences. However, shortly after being placed in conditions conducive to renaturation, most fold-back sequences will presumably form intramolecular duplexes as they do in vitro. This should effectively reduce potential hybridization of these sequences to chromosomal DNA.

A middle repetitive fraction probe containing DNA sequences with copy numbers between approximately 10 ($C_0t_{1/2}$ = 81.9) and 1000 ($C_0t_{1/2}$ = 0.819) was prepared using two successive HAP column DNA fractionations. The first fractionation (C_0t 0.819) was performed with no special adjustments, as total genomic DNA was used as the starting material. For the second fractionation, DNA with a $C_0t > 0.819$ that had been isolated during the first fractionation was dialyzed into TE buffer (pH 8.0), precipitated with ethanol, redissolved in 0.48 M SPB, denatured by heating, and reannealed to a C_0t equivalent to 81.9.[3] This DNA was run on a HAP column, and the DNA that eluted with 0.48 M SPB was saved for use as a middle-repetitive DNA probe. Because the middle-repetitive fraction was taken from an area of the C_0t curve adjacent to the highly repetitive DNA C_0t component, it is likely that this fraction is significantly contaminated by highly repetitive DNA. The middle-repetitive fraction presumably also contains roughly 10% of the single-copy DNA C_0t component.

Single-copy, middle-repetitive, and highly repetitive DNA fractions were labeled with biotin and used in FISH. As a positive control, the TGRI sequence of tomato was also hybridized to pachytene chromosomes.

Numerous pachytene chromosomes were examined, and a distinctive labeling pattern was observed for each of the probes (Fig. 5). Highly repetitive DNA sequences hybridized almost exclusively with pericentromeric heterochromatin and telomeric–subtelomeric regions of chromosomes (Figs. 5*a* and 5*b*). This pattern of labeling was observed using both the direct- and indirect-detection methods (see Materials and methods).

Using the direct-detection method, single-copy DNA strongly hybridized to heterochromatin and was only slightly visible in euchromatin. However, using indirect detection, single-copy DNA was readily detected in euchromatin (Figs. 5*c*–5*f*).

Likewise, the middle-repetitive DNA probe was observed almost exclusively in heterochromatin using direct detection, but amplification of hybridization signals using the indirect-detection method revealed some hybridization of the middle-repetitive DNA probe to euchromatin (Figs. 5*g* and 5*h*). Ganal et al. (1988) reported that the repeat sequences TGRII and TGRIII were interspersed along the entire length of some tomato chromosomes. Thus, some of the labeling of euchromatin by the middle-repetitive probe may be due to these interspersed elements. Because only 10% of the middle-repetitive probe is composed of single-copy DNA, it is probable that most labeling of euchromatin with this probe is due to the presence of middle-repetitive sequences in euchromatin. Since the middle-repetitive fraction is contaminated with highly repetitive sequences, it is unclear to what extent FITC staining of heterochromatin with the middle-repetitive probe is due to the actual presence of middle-repetitive sequences in heterochromatin.

The TGRI sequence hybridized to discrete sites along the chromosomes (in particular, to telomeric regions), as described by Lapitan et al. (1989) (Figs. 5*i* and 5*j*).

The highly specific and characteristic hybridization of the TGRI probe, coupled with the distinctive hybridization patterns of the repetitive and single-copy DNA probes, indicate that the FITC fluorescence observed in the repetitive and single-copy DNA preparations is not due to autofluorescence, nonspecific binding of the FITC conjugates to chromatin, or "bleed-through" of the DAPI counterstain. We interpret the results of FITC staining to indicate: (*i*) that practically all highly repeated sequences are located in pericentromeric heterochromatin and telomeric regions, (*ii*) that some middle-repetitive sequences are found in euchromatin and heterochromatin, and (*iii*) that while single-copy DNA comprises most of the DNA in euchromatin, heterochromatin contains the majority of single-copy DNA sequences, an observation consistent with our C_0t data and previous cytological study (Peterson et al. 1996).

Single-copy DNA and heterochromatin

In this study we demonstrate that approximately 73% of the tomato genome is composed of single-copy sequences. This finding is in agreement with earlier molecular studies (Ganal et al. 1988; Zamir and Tanksley 1988). Although such a high proportion of single-copy sequences is rather unusual among plants, *Arabidopsis* (1C = 0.17 pg; Galbraith et al. 1991) and mung bean (*Vigna radiata*, 1C = 0.47 pg) also have genomes composed primarily of single-copy DNA (Murray et al. 1981; Leutwiler et al. 1984; Pruitt and Meyerowitz 1986). Based upon a C_0t analysis of *Arabidopsis thaliana*, Leutwiler et al. (1984) suggested that the *Arabidopsis* genome is 86–90% single-copy DNA. Despite the high percentage of single-copy sequences in both tomato and *Arabidopsis*, tomato's genome is approximately 5 times larger than that of *Arabidopsis*. If *Arabidopsis* and tomato require a similar number of gene products, a significant portion of the tomato genome must be composed of nontranscribed single-copy DNA.

Because 77% of tomato chromosomal DNA is found in pericentromeric heterochromatin (Peterson et al. 1996) and the

[3] Because 15% of the genome had been removed from the DNA that had a C_0t value > 0.819, single copy number and low copy number sequences were more concentrated than they would have been in total genomic DNA. Thus, these sequences presumably could renature at a faster rate than would be expected if the starting material were whole genomic DNA. To reach the equivalent of a specific C_0t value when starting with an isolated C_0t fraction, the desired C_0t value must be multiplied by the fraction of the genome remaining single-stranded at the C_0t value of the starting material (see Hood et al. 1975). Using the techniques described in Material and methods, we determined that, for C_0t 0.819, this value was 85% ssDNA. Because the starting material for the second fractionation was DNA with a $C_0t > 0.819$, DNA with a C_0t equivalent to 81.9 was obtained by renaturing the DNA with a $C_0t > 0.819$ to a C_0t of (81.9 × 0.85 =) 69.3.

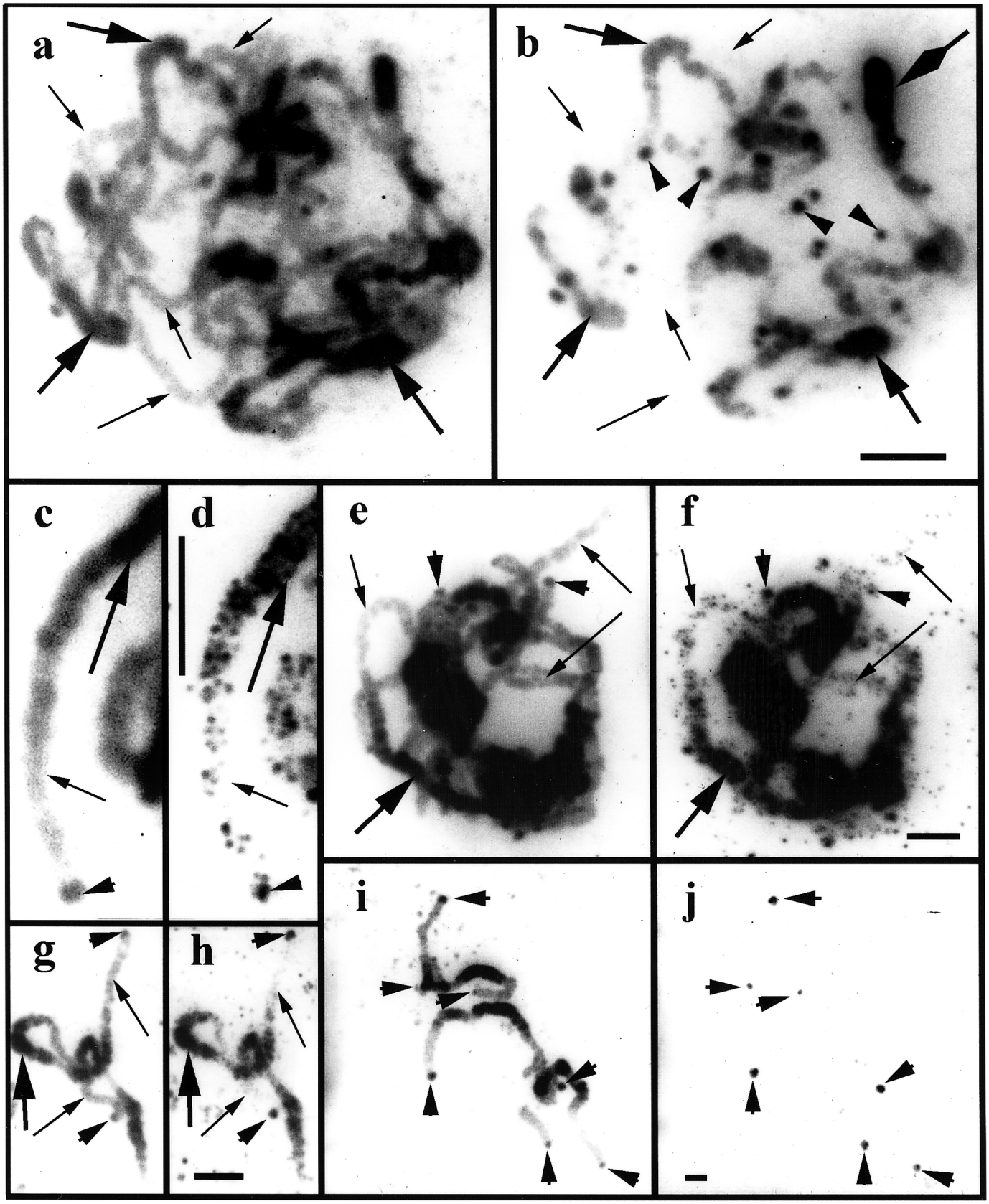


Fig. 5. FISH to pachytene chromosomes using highly repetitive DNA, single-copy DNA, middle-repetitive DNA, and the TGRI repeat sequence. All images have been reversed so that chromosomes appear dark on a light background. DAPI stained chromosome preparations are shown in *a*, *c*, *e*, *g*, and *i*, while corresponding FITC images (i.e., probe hybridization) are shown in *b*, *d*, *f*, *h*, and *j*, respectively. In all frames, small arrows indicate select regions of euchromatin, large arrows indicate select regions of heterochromatin, the diamond-head arrow indicates the nucleolus organizer region, and arrowheads indicate select telomeric–subtelomeric regions. Note that in the DAPI stained images, heterochromatin and euchromatin are clearly distinguishable based upon stain intensity (i.e., heterochromatin appears darker than euchromatin). The chromosomes shown in *a* and *b* have been probed with highly repetitive DNA (direct probe detection). Comparison of *a* and *b* reveals that highly repetitive DNA does not hybridize to euchromatic regions but intensely hybridizes with pericentromeric heterochromatin, the nucleolus organizer region, and telomeres. Hybridization of the single-copy probe (indirect detection) is shown in *c* through *f*. Comparison of each DAPI image with its corresponding FITC image reveals that while single-copy sequences hybridize to euchromatin, most single-copy DNA is found in heterochromatin. Telomeric regions are also recognized by the single-copy probe. The middle-repetitive probe was hybridized to the chromosomes shown in *g* and *h* (indirect probe detection). Comparison of the images shows that most middle-repetitive sequences are in heterochromatin, although some labeling of euchromatin is visible. The chromosomes shown in *k* through *n* have been probed with the TGRI repeat sequence (direct detection). Comparison of DAPI and FITC images shows that TGRI hybridizes to the subtelomeric regions of most chromosomes, as described by Lapitan et al. (1989). Scale bars = 5 μm.

tomato genome is primarily composed of single-copy DNA, pericentric heterochromatin of tomato must contain many single-copy sequences. Our FISH results indicate that this is true. Although this makes tomato heterochromatin rather atypical, there are reports of vertebrate species in which specific regions of heterochromatin are enriched in low copy number sequences (Comings and Mattoccia 1972; Arrighi et al. 1974). On the other hand, the highly-repetitive DNA of tomato appears to be confined to heterochromatin, a finding that suggests that tomato may be more tractable for chromosome walking studies and in situ localization of gene sequences (see below) than would be predicted based upon its genome size.

Implications for chromosomal in situ suppression (CISS) hybridization

During in situ hybridization, unlabeled repetitive DNA can be used to block repetitive sequences in large genomic probe molecules. This procedure is called CISS hybridization (Lichter et al. 1990) and it has proved an effective way to map genes using large clones as probes (e.g., Landegent et al. 1987; Lichter et al. 1988, 1990; Jiang et al. 1995). In recent years, it has become standard practice to "pre-anneal" genomic DNA probes with C_0t-1 DNA to suppress repetitive elements during in situ hybridization to chromosomes (see Zwick et al. 1997 for a review). However, as shown in this study, tomato possesses many low copy number repeats that would not be suppressed using C_0t-1 DNA. Such low copy number repeats theoretically could complicate CISS hybridization studies. In tomato, it is likely that the use of DNA with C_0t values ≥ 8.9 would be more effective in CISS hybridization than C_0t-1 DNA (although for tomato it is possible that the lack of repetitive DNA in euchromatin may permit in situ hybridization to genetic loci without supression of repetitive DNA). Likewise, it may benefit those interested in physical gene mapping in other species to review existing C_0t literature or to construct a C_0t curve for their species of interest prior to perfoming CISS hybridization.

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