Measurement of Unrestrained Negative Supercoiling and Topological Domain Size in Living Human Cells†

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ABSTRACT: Unrestrained DNA supercoiling was measured using a Me_3-psoralen photobinding assay within a transcriptionally active hygromycin B phosphotransferase (*hph*) gene integrated into different chromosomal locations in five transformed human fibrosarcoma cell lines. The level of unrestrained supercoiling in the *hph* gene varied, from high to low levels, in different chromosomal locations in living human cells. In one cell line, the *hph* gene contained no unrestrained supercoiling. Consequently, supercoiling was not dictated by the DNA sequence of the active *hph* gene. The addition of α-amanitin, which can inhibit transcription, reduced unrestrained supercoiling by 75% at one chromosomal location, by 50% at two other locations, and had little, if any, effect at two other chromosomal locations. Different levels of supercoiling in separate regions of the chromosome require that the chromosome be organized into independent topological domains *in vivo*. Evidence for independent topological domains in living cells is presented. From analysis of the relaxation of supercoiling as a function of the number of breaks introduced into the chromosome, the *in vivo* topological domain size for the human ribosomal RNA genes was estimated between 30 000 and 45 000 kb.

Unrestrained supercoiling observed in prokaryotic DNA is introduced by the activity of DNA gyrase and topoisomerase I [for reviews, see Gellert (1981) and Drlica (1992)], and localized changes in unrestrained supercoiling may be induced by transcription (Liu & Wang, 1987; Rahmouni & Wells, 1989; Zheng et al., 1991). While, on average, bulk eukaryotic chromatin is relaxed (Sinden et al., 1980), the few gene coding regions analyzed to date are organized with unrestrained supercoiling (Leonard & Patient, 1991; Ljungman & Hanawalt, 1992, 1995; Jupe et al., 1993, 1995). The processes responsible for the introduction of unrestrained supercoils in eukaryotic DNA are not well understood. These processes may include: (1) the release of restrained DNA supercoils by the loss of nucleosomes or other chromosomal components which influence the writhe (Esposito & Sinden, 1988; Freeman & Garrard, 1992), (2) unfolding nucleosomes (Prior et al., 1983; Chen & Allfrey, 1987), (3) enzymatic supercoiling (Ohta et al., 1995), or (4) movement of RNA polymerase (Brill & Sternglanz, 1988; Giaever & Wang, 1988; Osborne & Guarente, 1988). Histone acetylation has been associated with gene activity, but there are conflicting reports as to its influence on writhe (Norton et al., 1990; Lutter et al., 1992; Bauer et al., 1994).

The existence of unrestrained negative supercoiling, and especially the localization of unrestrained supercoiling in eukaryotic cells, is dependent on the partitioning of DNA into loops, which may delimit the boundaries of topologically closed domains [see for discussion Freeman and Garrard (1992)]. *In vitro* studies have identified A+T-rich regions in DNA which may be the sites that attach DNA onto the nuclear matrix or scaffold (MARs, SARs) and define topological domains (Cockerill & Garrard, 1986; Gasser & Laemmli, 1986; Gross & Garrard, 1987). Several other factors such as topoisomerases, transcription or replication complexes, or specialized chromatin structural elements may also define a looped region or a topological domain within a looped region. All such chromatin boundaries are termed "functional" in nature (Jackson et al., 1992). Data from a variety of *vitro* techniques suggest that chromosomal loop sizes vary considerably from less than 1 kb to 300 kb per domain (Benyajati & Worcel, 1976; Hofmann et al., 1989; Jackson et al., 1990).

The correlation between unrestrained DNA supercoiling and transcription activation suggests an important role for supercoiling in transcriptional initiation (Leonard & Patient, 1991; Dunaway & Ostrander, 1993; Parvin & Sharp, 1993; Ljungman & Hanawalt, 1995). Negative superhelical energy facilitates the assembly of the RNA polymerase II transcription complex *in vitro* (Tabuchi & Hirose, 1988; Mizutani et al., 1991; Parvin & Sharp, 1993), and the enhancement of ribosomal RNA transcription by negative unrestrained supercoiling has been observed (Schultz et al., 1992). This suggests a role for superhelical energy in the initiation of transcription. Furthermore, relaxing supercoils by nicking DNA with X-rays *in vivo* leads to the inhibition of transcription, again suggesting a role for unrestrained supercoiling in transcription (Luchnik et al., 1988).

In the *Drosophila* locus 87A7 containing two *hsp*70 genes, unrestrained supercoiling exists constitutively over a localized chromosomal region (Jupe et al., 1993, 1995). High levels of negative supercoiling were detected within the coding region of the *hsp*70 gene and within the *scs* and *scs′* sequences that define the 87A7 microdomain boundaries. Intermediate levels of supercoiling exist in DNA regions flanking the *hsp*70 coding region, while DNA distal to the *scs* sequence is devoid of unrestrained supercoiling. A high

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level of unrestrained supercoiling was constitutive over the coding region irrespective of the transcriptional state of the gene, although supercoiling was increased by about 20% upon gene induction by heat shock. At present, the biological significance of unrestrained supercoiling is uncertain, as is the nature of the molecular signals that regulate maintenance of differential levels of supercoiling within a defined chromosomal region. Moreover, it is unknown if localized unrestrained supercoiling differs between different topological regions, if supercoiling varies during the cell cycle, or if different levels of unrestrained supercoiling can be associated with DNA during cellular processes such as transcription or replication. To begin to address some of these questions, we have measured supercoiling *in vivo* within a transcriptionally active gene integrated randomly at several chromosomal locations in human cells. The results suggest that unrestrained supercoiling may not be a prerequisite or requirement for transcription, nor does transcription appear to be the only factor responsible for the generation of unrestrained supercoils. In addition, the topological domain size for the rDNA in living human cells is estimated by analyzing the loss of supercoiling as a function of the number of nicks introduced into the genome.

EXPERIMENTAL PROCEDURES

Construction and Production of Plasmids. Three plasmids, pMC1nF14C, pMC1n122, and pMC1nh, were derived from pMC1neopA (Stratagene) which contains a *neo*[1] gene driven by the MC1 promoter. pMC1nF14C was constructed by the initial addition of a 106 bp inverted repeat termed F14C, which was derived from pBR325F14C (Zheng & Sinden, 1988) by digestion with *Eco*RI. To allow insertion of the F14C fragment, *Nco*I−*Eco*RI 11 bp linkers (5′GATGGAGCTCC3′ and 5′AATTGGAGCTC3′) were ligated onto the 106 bp F14C fragment to create the 122 bp perfect inverted repeat, F14CE, and this was inserted into the *Nco*I site within the *neo* gene of pMC1neopA. pMC1n122 was constructed by the initial addition of a nonpalindromic 122 bp sequence, which was produced from pBR322 by PCR using primers 5′CATGCCATGGGCTCCAACGTGAGCATCCTCTCTCGTTTCATC3′ and 5′CATGCCATGGAGCTCCAAGGGCGGTTTTTTCCTGTTTGGTCAC3′. The PCR fragment was inserted into the *Nco*I site in the *neo* gene of pMC1neopA. Construction of both pMC1nF14C and pMC1n122 was completed by adding a *Bam*HI fragment from pSV2*HPH* (American Type Culture Collection) containing the hygromycin B phosphotransferase gene (*hph* driven by an SV40 promoter), which was inserted into the *Bam*HI site of each plasmid. pMC1nh was made by inserting the *Bam*HI fragment containing the hygromycin B phosphotransferase gene into pMC1neopA. Restriction endonucleases were purchased from either Bethesda Research Laboratories or New England Biolabs, and digestions were performed as recommended by the supplier.

Plasmids pMC1nh, pMC1nhF14C, and pMC1nh122 were transformed into DH5α. Transformed cells were grown in Luria broth, and the plasmid DNA was isolated by a cleared lysate procedure (Sinden et al., 1980). Plasmids were banded twice in CsCl−ethidium bromide gradients. Ten micrograms of this DNA was EtOH-precipitated twice and air-dried under sterile conditions for use in the electroporations.

Cell Culture and Generation of Cell Lines. HTD114 cells (Liu et al., 1989), derived from HT-1080 (Rasheed et al., 1974), were grown on 100 mm plates in minimal essential medium (MEM) with 7.5% horse serum and 2.5% fetal bovine serum (Sigma) in 5% CO_2. The cells were grown to a density of $(3-5) \times 10^6$ per plate and washed twice with phosphate-buffered saline (PBS), and 0.5 mL of trypsin (0.25%) was added. The cells were harvested, counted, and centrifuged at 100g for 10 min. The cells were then resuspended in MEM plus serum at 2.4×10^6 cells/mL. Ten micrograms of *Xho*I-digested, linear plasmid DNA was resuspended in 200 μL of MEM medium with serum and mixed with 800 μL of the diluted cells. The mixture was added to a chilled 0.4 cm cuvette and electroporated using 960 mF and 200 V/cm (Bio-Rad Gene Pulser). The electroporated cells were mixed in 24 mL of MEM with serum, and 1 mL of this solution was added to each well of a 24-well plate (Corning). Twenty four hours after the transformed cells were plated, the medium was removed, and fresh medium containing 100 units/mL hygromycin (Sigma) was added. The medium was changed daily, and after 10−14 days, hygromycin-resistant colonies were visible. Each colony was picked, plated, and grown for freezing. Southern analysis was performed as described previously (Lupski et al., 1991) to determine the *hph* gene constructs (Figure 1) relative location and copy number.

Nicking and Cross-Linking Assays. Two 100 mm plates were started from a frozen culture of cells and grown to a density of 5×10^6 cells/plate. The cells were washed twice in PBS, and then 0.5 mL of trypsin (0.25%) per plate was added. The cells were resuspended to a density of 1×10^6 cells/mL in MEM medium with serum, 10 mL of media was added to each of 9 plates (1×10^6 cells/plate), and 10 mM bromo-2-deoxyuridine (BrdUrd) was added. Using this protocol in hamster cells, 45% of the thymidines were substituted with BrdUrd (Kaufman & Davidson, 1978). The cells were grown to a density of $(7-8) \times 10^6$ cells/plate. For α-amanitin experiments, 0.4 μg/mL α-amanitin was added to the media 24 h prior to performing the cross-linking assay. The cells (attached to plate) were washed twice with 20 mL of PBS buffer, 2 mL of PBS buffer was added, and the plates were chilled to 0 °C. To introduce nicks into DNA, cells were exposed to 313 nm light using a mercury vapor lamp with a K_2CrO_4/NaOH filter (Sinden & Pettijohn, 1982; Sinden & Ussery, 1992). To the nicked and nonnicked samples was added 20 μL of a saturated 4,5′,8-trimethylpsoralen (Me_3-psoralen)/EtOH solution, and after 5 min incubation at 0 °C, the plates were exposed to various doses of 360 nm light (Sinden & Pettijohn, 1982; Sinden & Ussery, 1992). After treatment, the cells were washed twice with PBS, harvested, pelleted, and resuspended in 3 mL of extraction buffer (200 mM Tris, pH 8.0, 200 mM NaCl, 100 mM EDTA, and 4% SDS), 50 μL of RNase A (10 mg/mL) was added, and the mixture was incubated at 37 °C for more than 8 h. Then 125 μL of proteinase K (20 mg/mL) was added, and following an 8 h incubation at 55 °C, the protein was removed by one extraction with 3 mL of phenol equilibrated with 10 mM Tris, pH 7.6, 50 mM NaCl, and 1 mM EDTA (TEN) and one extraction with 3 mL of 24:1 chloroform/isoamyl alcohol. The DNA was precipitated by addition of 0.1 volume of 3 M potassium acetate and 2.5

[1] Abbreviations: *hph*, hygromycin B phosphotransferase gene; *neo*, neomycin resistance gene; Me_3-psoralen, 4′,5′,8-trimethylpsoralen; BrdUrd, 5-bromo-2-deoxyuridine.

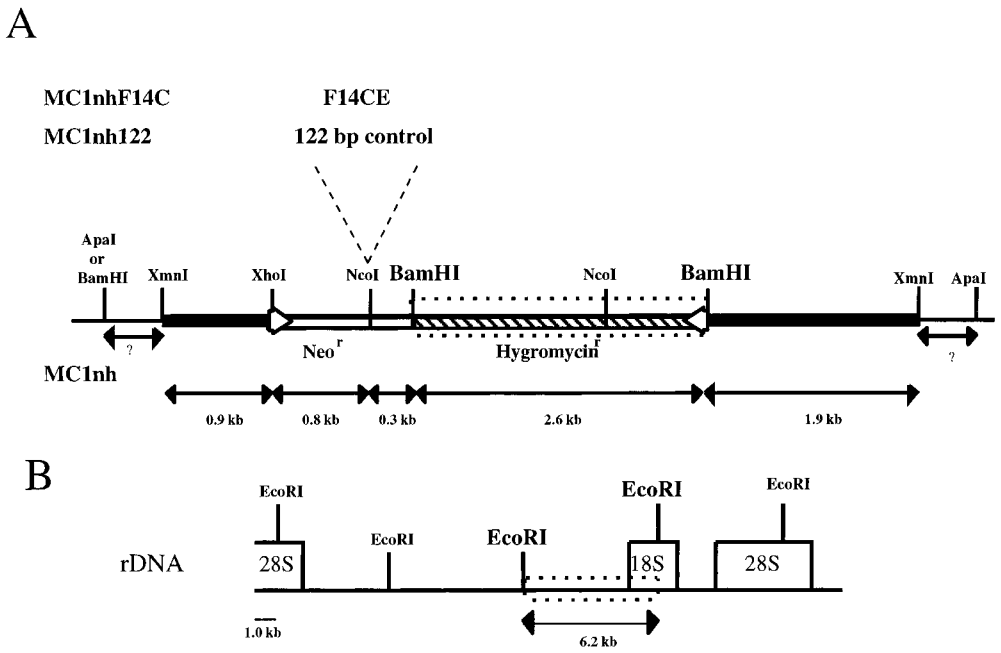

FIGURE 1: Chromosomal map of the hygromycin gene and ribosomal gene unit. (A) Derivatives of plasmid MC1nh (thick line) containing the inverted repeat F14CE and a 122 bp nonpalindromic sequence in the *Nco*I site of the *neo* gene (thin white box) are termed pMC1nhF14CE and pMC1nh122, respectively. These plasmids were transformed as linear *Xmn*I fragments into a human fibrosarcoma cell line (HTD 114). The *hph* gene is denoted by the thin hatched box. The 2.6 kb *Bam*HI fragment (dotted box) was used as a hybridization probe. (B) Map of a single, complete ribosomal DNA gene unit from the human chromosome. 18S and 28S represent the genes leading to the production of the ribosomal subunits. The 6.2 kb *Eco*RI fragment (bounded by a dotted box) was used as a hybridization probe.

volumes of 95% ethanol. The samples were resuspended in 10 mM Tris, pH 7.0, 5 mM EDTA at a concentration of approximately 300 μg/mL.

Southern Analysis of Photo-Cross-Linked DNA. Seven micrograms of chromosomal DNA was digested to completion at 37 °C with 100 units of restriction endonucleases in a 60 μL volume. After digestion, 300 μL of TE was added to the digests, and two extractions with 100 μL of TEN-saturated phenol and two extractions with 100 μL of chloroform/:isoamyl/alcohol (24:1) were performed. The samples were ethanol-precipitated, pelleted, dried, and then resuspended in 9 μL of H_2O. The samples were boiled and treated simultaneously with glyoxal aldehyde (6% of the total volume) and dimethyl sulfoxide (DMSO) (70% of the total volume) for 1 h at 50 °C. The treated samples were separated on a 1% agarose gel in 10 mM sodium phosphate (pH 7.0) as described (McMaster & Carmichael, 1977). Once electrophoresis was complete, the gel was treated for 3 h in denaturing solution (0.5 M NaOH, 1.5 M NaCl). The DNA was transferred to a nylon membrane (Genescreen plus, Dupont) and then dried at 80 °C in vacuum for 2 h. A 2.6 kb *Bam*HI fragment (hygromycin gene) was isolated from plasmid pMC1nh to use as a probe for detecting the hygromycin phosphotransferase gene. The membrane was hybridized with a [α-^{32}P]dCTP-labeled probe, labeled by the random priming method (Boehringer Mannheim). Quantitation was completed using a Molecular Dynamics PhosphorImager using ImageQuant software.

The fraction of DNA cross-linked (F_x) was calculated by dividing the area of the cross-linked peak by the sum of the area for the cross-linked and non-cross-linked peaks. Cross-links per kilobase (X_l/kb) values were calculated using the formula $X_l/\text{kb} = -\ln(1 - F_x)/S$, as previously described (Jupe et al., 1993), where S is the size of the restriction fragment [S = 2.6 kb for the *hph* probe and 6.2 kb for the ribosomal RNA gene (rDNA) probe]. The ratio of the mean cross-linking rate in intact verses relaxed domains is referred to as the *relative superhelical density*, $R_{I/N}$, where $R_{I/N} = (X_l/\text{kb}_I)(X_l/\text{kb}_N)$. $R_{I/N}$ reflects the level of unrestrained supercoiling *in vivo*, and although the rate of cross-linking is linearly proportional to the negative superhelical density (Sinden et al., 1980), a definitive level of supercoiling cannot be ascribed to a particular $R_{I/N}$ value as discussed by Jupe et al. (1993). However, a $R_{I/N}$ value of 1.35 may suggest a superhelical density of $\sigma = -0.015$ to -0.03, although this needs to be determined with a quantitative assay. To determine an $R_{I/N}$ value, an average of two Southern blots was analyzed for a minimum of four separate experiments for most analyses. A single Southern blot was analyzed for two of the four experiments completed for cell lines 122-2, 122-6, and F14C-4 treated with α-amanitin, but two Southern blots were analyzed for the two remaining experiments, as mentioned above. An unpaired, two-tailed, *t*-test was performed with these $R_{I/N}$ values, and a significant difference is indicated if $P \leq 0.05$.

While there is a linear relationship between the rate of psoralen photobinding and negative superhelical density (Sinden et al., 1980), the behavior of this relationship for positively supercoiled DNA is not known. Therefore, at present a value of $R_{I/N} < 1$ cannot be assumed to reflect positively supercoiled DNA.

Measurement of Single-Strand Breaks. To measure the frequency of single-strand breaks introduced by BrdUrd photolysis, cells were grown 3−4 generations in the presence of 0.2 mCi of [^{3}H]thymidine before the DNA was nicked as described above. Following nicking, the cells were washed in 3 mL of PBS buffer and pelleted. The cells were resuspended in 1 mL of PBS and placed on ice. A total of (1−5) × 10^4 cells and 2000 cpm of ^{14}C-labeled T7 phage were added to 0.1 mL of 1 M NaOH, 10 mM EDTA layered

on an alkaline sucrose gradient (5−20% sucrose in 0.3 M NaOH, 2 M NaCl, and 10 mM EDTA). After loading, the gradients were put in the dark at 20 °C for 3 h before sedimentation at 30 000 rpm in a Beckman SW55Ti rotor for 2−4 h. Thirty equal volume fractions were collected from the bottom of the tube onto 2 cm sections of a 0.5 in. strip of Whatman 3MM filter paper. Once dry, the paper was washed twice in 5% trichloroacetic acid, and once in 95% EtOH. After drying, the 2 cm sections were placed into vials with scintillation fluid and counted for radioactivity. Molecular weights of the single-strand DNA fragments were calculated as described by Rupp and Howard-Flanders (1968). The number of single-strand breaks per genome was calculated using a molecular mass of 338 g/mol base and a modal number of 46 chromosomes per cell (Rasheed et al., 1974) with 7.8×10^9 bp/genome.

RESULTS

Cell Lines. The five cell lines used in this study were made by introducing the hygromycin phosphotransferase gene (*hph*) and a modified *neo* gene, in convergent orientations, into the chromosome of a human fibrosarcoma cell line, HTD 114 (Liu et al., 1989). The modified *neo* gene contained either a 122 bp nonpalindromic or palindromic insert cloned into the *Nco*I site, creating G-418-sensitive cell lines designated as 122- or F14C-lines, respectively. The "mutation insert" in the *neo* gene allowed measurement of the rate of deletion of the insert for the *neo* gene (Kramer et al., 1996). The level of unrestrained supercoiling was measured in three cell lines which contain an insertion of pMC1nhF14CE (with the inverted repeat insert) and two cell lines which contain an insertion of pMC1nh122 (with the nonpalindromic insert), all in different chromosomal locations (Figure 1A). Southern analysis of chromosomal DNA probed with a 2.6 kb fragment (*hph*) indicated a single complete insertion in four cell lines: F14C-8, F14C-4, 122-2, and 122-6, and two *hph* genes in cell line F14C-23 (data not shown). Cell line F14C-4 does not have a complete 5′ region between the *Xho*I and *Nco*I sites of the *neo* gene (Figure 1A) as determined by PCR (data not shown) and, thus, may represent an *hph* gene that is not adjacent to an active convergent *neo* gene. In the case of cell lines F14C-23, 122-2, and 122-6, *neo*-resistant revertants of these cell lines can be isolated, indicating that the *neo* gene is transcribed in these cell lines (Kramer et al., 1996).

Rate of Me_3-Psoralen Cross-Linking in a Hygromycin Gene Varies at Different Chromosomal Locations. Cross-linking rates for a 2.6 kb *Bam*HI fragment containing the *hph* gene were determined for each chromosomal location using the five transformed cell lines. An advantage in using the five cell lines with the same insertion is that since the identical sequence is being analyzed in each cell line, the cross-linking rates will not be influenced by base sequence. Therefore, any change in the cross-linking rate should reflect the inherent affinity of that chromosomal location to the intercalation of Me_3-psoralen due to accessibility and/or unrestrained supercoiling. As discussed by Sinden and Ussery (1992), the rate of psoralen photobinding is dependent upon the extent of association with nucleosomes, or other proteins that prevent Me_3-psoralen binding, and the level of unrestrained supercoiling (Jupe et al., 1993). The cross-links per kb (X_l/kb) increased linearly with light dose to at least 8 kJ/m^2 for all five cell lines tested (Figure 2). The individual rates of cross-linking, however, varied approximately 2-fold between two cell lines (122-6, F14C-8) and F14C-23. The cross-linking rates in the *hph* gene of cell lines 122-2 and F14C-4 were nearly equal.

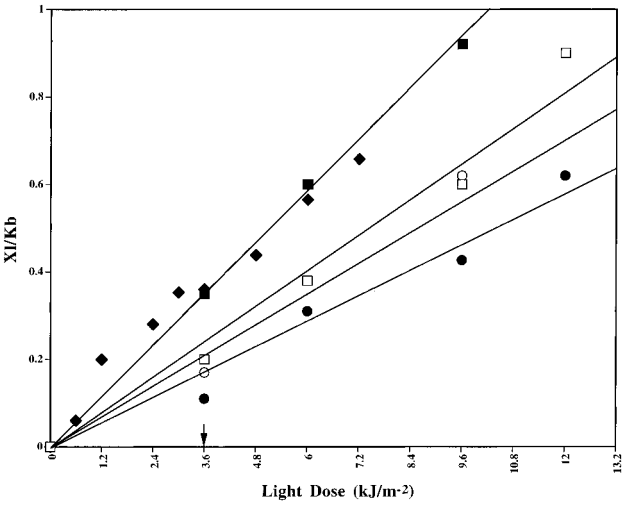

FIGURE 2: Rates of cross-linking the *hph* gene in the HTD 114 cell lines. Cells were treated with 360 nm light at an incident intensity of 1.2 kJ m^{-2} min^{-1}. Samples were removed after various times and analyzed by Southern hybridization to determine the cross-links per kb (X_l/Kb) as described under Experimental Procedures. (■) 122-6; (◆) F14C-8; (□) 122-2; (○) F14C-4; (●) F14C-23. The arrow at 3.6 kJ/ m^2 indicates that the dose at which the $R_{l/N}$ values were determined is within the linear range of this cross-linking reaction.

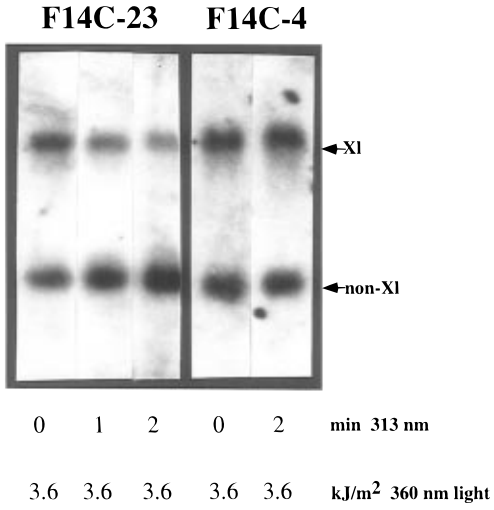

FIGURE 3: Southern analysis of intact and nicked chromosomal DNA. Southern blot analysis was performed on DNA isolated from cell lines with pMC1nhF14C or pMC1nh122 randomly inserted. Cells were exposed to 0, 1, or 2 min of 313 nm light, treated with Me_3-psoralen, and then exposed to 0 or 3.6 kJ/m^2 360 nm light. (Xl) indicates the cross-linked species, and non-Xl indicates the non-cross-linked species of the 2.6 kb *Bam*HI fragment.

Different Levels of Unrestrained Supercoiling Exist in Hygromycin Genes Integrated at Different Chromosomal Locations. Representative Southern blots of two cell lines exposed to Me_3-psoralen and 3.6 kJ/m^2 of 360 nm UV light with and without nicking are shown in Figure 3. A dose of 3.6 kJ/m^2 of 360 nm light was used to determine the level of unrestrained negative superhelical energy because it is within the linear range of the cross-linking reaction (Figure 2). DNA was nicked *in vivo* by a 1 or 2 min exposure to

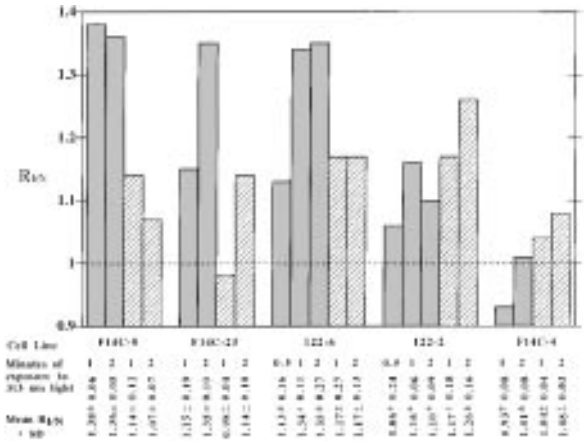

FIGURE 4: $R_{I/N}$ values for the five cell lines. $R_{I/N}$ values were calculated as described under Experimental Procedures from multiple experiments, as shown in Figure 3. A dashed line at $R_{I/N} = 1$ represents no unrestrained supercoiling. $R_{I/N} > 1$ indicates the presence of unrestrained negative supercoiling. The shaded bars are the $R_{I/N}$ value for the cells without addition of α-amanitin, and the striped bars are the $R_{I/N}$ value for the cells treated with α-amanitin 24 h prior to psoralen photobinding. The data represent at least four separate experiments of which two Southerns were performed.

313 nm light before Me_3-psoralen cross-linking. A substantial decrease in the fraction of cross-linked DNA fragments was detected in DNA from cell line F14C-23 after nicking by 1 or 2 min exposure to 313 nm light (Figure 3). This decrease following nicking is diagnostic for the presence of unrestrained supercoiling (Jupe et al., 1993). DNA from cell line F14C-4 showed no change in the fraction of cross-linked fragments after nicking for 2 min, suggesting that no unrestrained supercoiling was present (Figure 3).

The relative superhelical density ($R_{I/N}$) was calculated from the cross-links per kb values determined from analyses of multiple, independent Southern blots. A ratio of $R_{I/N} = 1$ indicates completely relaxed DNA while a ratio of $R_{I/N} > 1$ indicates the presence of unrestrained supercoiling. The level of unrestrained supercoiling detected in the *hph* gene was different in different chromosomal locations as shown in Figure 4. In cell lines F14C-8, F14C-23, and 122-6, similar levels of negative unrestrained supercoiling ($R_{I/N} = 1.34-1.38$) were indicated after 2 min of nicking (Figure 4, shaded bars). The *hph* gene in cell line 122-2 had a low level of negative unrestrained supercoiling whereas the gene in cell line F14C-4 was devoid of any unrestrained supercoiling as indicated by an $R_{I/N} \leq 1$ (Figure 4). The differences in the level of supercoiling, as indicated by different $R_{I/N}$ values, between cell line F14C-8 and cell lines 122-2 and F14C-4 were statistically significant ($P \leq 0.05$) (see Experimental Procedures). Also, a significant difference in the level of supercoiling existed between cell lines 122-6 and F14C-4.

Influence of α-Amanitin on Supercoiling. The rate of Me_3-psoralen photobinding in each of the five cell lines was analyzed 24 h after the addition of α-amanitin (0.4 mg/mL) to the media. This concentration of α-amanitin inhibits about 90% of transcription by eukaryotic RNA polymerase II *in vivo* (Jendrisak, 1980). A significant decrease in the $R_{I/N}$ value in cell line F14C-8, from 1.36 to 1.07 after 2 min nicking, was observed when the cells were treated with α-amanitin (striped bars, Figure 4). In addition, a large change from 1.35 to 1.14 and 1.17 was observed in cell lines F14C-23 and 122-6, respectively. The level of unrestrained supercoiling in cell line 122-2 appeared consistently higher following α-amanitin treatment. Cell line F14C-4 had no detectable unrestrained supercoiling with (striped bar) or without α-amanitin (shaded bar) (Figure 4).

Measurement of a Topological Domain Containing the Ribosomal RNA Genes in Living Cells. The size of a topological domain containing unrestrained DNA supercoiling in living cells can be estimated by measuring the number of nicks required to relax supercoiling in cells (Sinden & Pettijohn, 1981). The fraction of the original unrestrained supercoiling remaining after the introduction of a defined number of nicks can be described by the zero-order term of the Poisson distribution, $F_R = p(0) = e^{-(x/m)}$, where x is the number of nicks introduced in the genome and m is the number of domains in the genome (Sinden & Pettijohn, 1981, 1982). m can be estimated from the best fit to the experimental data. This method assumes that nicks are distributed among the topological domains in a Poisson distribution.

To measure a topological domain size, the relative superhelical density ($R_{I/N}$) was measured in the multiple copies of the ribosomal RNA genes of cell line HTD 114 since previous studies have demonstrated that the ribosomal genes are wound with a high level of unrestrained supercoiling (Ljungman & Hanawalt, 1992; Jupe et al., 1993). Unrestrained supercoiling within a 6.2 kb *Eco*RI rDNA fragment (Figure 1B) was analyzed using this fragment, isolated as a probe from pU6.2 (Dante et al., 1992). The level of unrestrained supercoiling for the rDNA decreased to $R_{I/N} = 1.38$ following 5 min of nicking. This $R_{I/N}$ value is lower than the values observed in *Drosophila* (Jupe et al., 1993) and in a human cell line (Ljungman & Hanawalt, 1992). It is conceivable that this represents a maximal level of supercoiling in this cell line, a level that is lower than that observed in other cell types.

The fraction of DNA supercoils remaining following nicking (F_R), calculated using the $R_{I/N}$ values, is plotted against the number of breaks per genome equivalent introduced into the DNA in Figure 5. The number of nicks per genome equivalent of DNA was determined from alkaline sucrose gradient sedimentation analysis which provides an average molecular weight of single-stranded DNA (Rupp & Howard-Flanders, 1968). A linear relationship existed between the number of breaks in the double-stranded genomic DNA and the 313 nm light dose following nicking doses of 30 s or greater (insert of Figure 5). Figure 5 also shows the theoretical curves for F_R, calculated using different values for domain size (m). The experimental data for the rDNA genomic region best fit the theoretical curves having 200 000 (Figure 5, curve E) or 300 000 (Figure 5, curve F) domains per genome which corresponds to 39 000 and 26 000 bp per domain respectively, using a value of 7.8×10^9 bp per genome. Assuming that 10−20% of the nicks observed on alkaline sucrose gradients are alkali-labile breaks (not true breaks *in vivo*), as was observed for *E. coli* DNA *in vivo* (Krasin & Hutchinson, 1978), the domain size estimates should be proportionally higher, i.e., 30 000 ± 1 500 and 45 000 ± 2 000 bp per domain. The domain size calculation also assumes that all rDNA genes in the multiple loci are nicked equally for a given dose of 313 nm light and that the rate of nicking in these genes reflects the average rate of nicking for the entire chromosome.

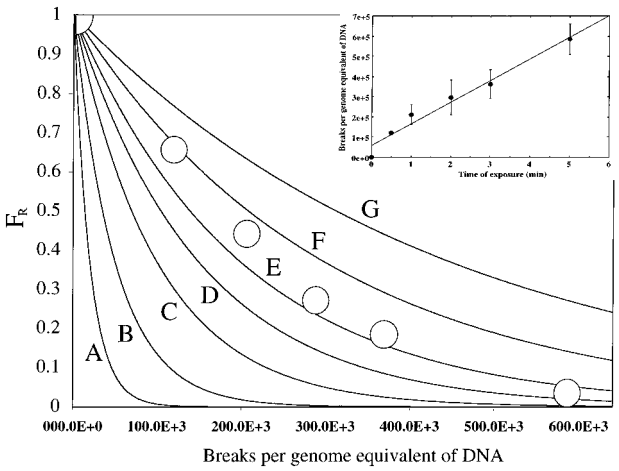

FIGURE 5: Estimation of the domain size of the ribosomal RNA gene region. Experimental data points are plotted as F_R, the fraction of total supercoils remaining after various times of nicking, vs the number of breaks introduced into the genome. F_R values are calculated from the $R_{I/N}$ values as $F_{Rx} = (R_{I/N}, 5\ min - R_{I/N}, X\ min)/(R_{I/N}, 5\ min - R_{I/N}, 0\ min)$, where $R_{I/N}, 0\ min = 1$. $R_{I/N}$ values of 1, 1.12, 1.21, 1.28, 1.31, and 1.38 for 0, 0, 5, 1, 2, 3, and 5 min of nicking resulted in F_R values of 1, 0.67, 0.46, 0.24, 0.16, and 0, respectively. (Insert graph) The number of breaks per genome equivalent are plotted against the time of exposure to 313 nm light. The molecular weight of the single-stranded DNA fragments was determined by alkaline sucrose gradient sedimentation analysis. The breaks per genome equivalent of DNA were calculated using a value of 7.8×10^9 bp/genome. Theoretical curves were calculated using the formula $F_R = e^{-(x/m)}$ using different values of "*m*" (domains per genome equivalent) and breaks per genome equivalent (*x*). The curves correspond to 20 000 (curve A); 50 000 (curve B); 100 000 (curve C); 150 000 (curve D); 200 000 (curve E); 300 000 (curve F); and 450 000 (curve G) domains per genome equivalent.

DISCUSSION

Measurements of the rate of Me_3-psoralen photobinding and cross-linking have provided a sensitive way to detect a relative superhelical density in both prokaryotes and eukaryotes (Sinden et al., 1980; Ljungman & Hanawalt, 1992, 1995; Jupe et al., 1993) and to measure topological domains in bacteria and bacteriophage DNA (Sinden & Pettijohn, 1981, 1982). Here we use this assay to analyze localized unrestrained supercoiling in a gene integrated at different chromosomal locations. The relative superhelical density found in the chromosomal locations in cell lines 122-6, F14C-23, and F14C-8 had similar $R_{I/N}$ values of 1.36−1.35 which was greater than the relative superhelical density found in cell lines 122-2 and F14C-4 (Figure 4). These data demonstrate that differences in the level of unrestrained supercoiling can exist in different chromosomal locations in a living human cell. Moreover, they demonstrate that supercoiling is not dictated by DNA sequence of the active *hph* gene. The similar level of unrestrained supercoiling present in the *hph* genes in cell lines F14C-23, F14C-8 and 122-6 and the 2-fold lower overall rate of cross-linking in F14C-23 compared to F14C-8 and 122-6 are consistent with a greater density of nucleosomes on the *hph* gene in cell line F14C-23.

The different levels of unrestrained supercoiling observed in the *hph* gene in the different chromosomal locations can be explained by several models. Supercoiling may be induced by proteins in combination with topoisomerase II (Ohta et al., 1995), and differences in the level of supercoiling in particular chromosomal domains could be explained by different enzymatic activities or differential regulation at specific domains by different topoisomerase II isozymes (Chung et al., 1989). A particular chromosomal domain may have specific DNA sequences at the domain boundaries *in vivo* (Adachi et al., 1989; Sperry et al., 1989) which may allow the differential binding of topoisomerase II isozymes (Drake et al., 1989) or differential activity (Boege et al., 1993). Topoisomerase recognition may also involve other factors such as Z-DNA which can influence the binding specificity of topoisomerase II (Glikin et al., 1991; Arndt-Jovin et al., 1993). Variation in unrestrained supercoiling could also be a byproduct of different levels of transcription, resulting from differential chromatin organization of the SV40 promoter driving the hygromycin gene or from differences in transcriptional activation (Patient & Allan, 1989).

Influence of α*-Amanitin on Unrestrained Supercoiling.* α-Amanitin had different effects on the level of negative unrestrained supercoiling within the *hph* gene in the different chromosomal locations. The low concentration of α-amanitin used (0.4 mg/mL) inhibits about 90% of transcription by eukaryotic RNA polymerase II *in vivo* (Jendrisak, 1980). In cell line F14C-8, a 75% decrease in the level of supercoiling was observed following treatment with α-amanitin. The level of negative unrestrained supercoiling was reduced by about half in cell lines F14C-23 and 122-6 when exposed to α-amanitin (Figure 4). In these cell lines, transcription may be responsible for maintenance of some, but not all, superhelical energy in certain chromosomal locations. This conclusion is supported by the observation that a 15−20% higher level of unrestrained supercoiling was observed in *Drosophila hsp*70 genes upon activation of transcription by heat shock (Jupe et al., 1993). The low level of unrestrained supercoiling in cell line 122-2 that was unaffected by α-amanitin and the similar levels of supercoiling in F14C-8, F14C-23, and 122-6 after α-amanitin treatment are consistent with the idea that a constitutive level of unrestrained supercoiling can exist in some regions of the DNA, independent of transcription (Leonard & Patient, 1991). In Chinese hamster ovary cells, supercoiling localized over the promoter in the dihydrofolate reductase gene promoter was not dependent on transcription elongation, but likely activation for transcription (Ljungman & Hanawalt, 1995). α-Amanitin did not decrease the level of negative unrestrained supercoiling detected in the rDNA genes which are transcribed by the α-amanitin-insensitive RNA polymerase I (data not shown). These results are consistent with the hypothesis that transcription by RNA polymerase II can influence the level of negative supercoiling in certain regions of DNA in some topological domains, but that transcription *per se* is not a sole determinant for the introduction of unrestrained supercoiling.

On average, bulk eukaryotic chromatin lacks unrestrained supercoiling (Sinden et al., 1980). A lack of unrestrained supercoiling was observed in a chromosomal region outside the functional *hsp* 70 domain in *Drosophila*, in contrast to a transcriptionally induced and elongating region which contained unrestrained supercoiling (Jupe et al., 1995). The *hph* gene integrated into cell line F14C-4 had no detectable unrestrained supercoiling. This cell line is resistant to hygromycin, which indicates the hygromycin phosphotransferase gene is active. A feature unique to this cell line is that the adjacent, convergent *neo* gene may not have a

complete promoter sequence 5′ to the coding region (between the *Xho*I and *Nco*I sites). The exact nature of the 5′ sequence difference is unknown, but a PCR product was not produced using a primer near the *Xho*I site which is near the promoter. A deletion at the promoter of the *neo* gene could affect the supercoiling within this chromosomal region since convergent transcription units may no longer be present. Alternatively, the relaxed state of the DNA may also be due to loss of a transcriptional activation step for the *neo* gene which induces supercoiling. This is the first reported case of a transcriptionally active chromosomal domain lacking unrestrained supercoiling. This locus may represent a type of chromosomal location that does not require unrestrained supercoiling for transcriptional activation, which is consistent with a study in *Rhodobacter capsulatus* in which no negative supercoiling was associated with transcription (Cook et al., 1989). In contrast, evidence *in vitro* and *in vivo* suggests that supercoiling facilitates transcriptional activation in certain prokaryotic and eukaryotic genes (Pruss & Drlica, 1989; Dunaway & Ostrander, 1993; Parvin & Sharp, 1993; Ljungman & Hanawalt, 1995). The absence of unrestrained supercoiling in the transcriptionally active region of F14C-4 suggests that not all transcriptionally active sequences have or need a constitutive level of unrestrained supercoiling.

Measurement of Topological Domains in Living Human Cells. Many approaches have been used to measure the size of the topological domains in eukaryotic chromosomes with estimates of between 1 and 300 kb per domain (Benyajati & Worcel, 1976; Hofmann et al., 1989; Jackson et al., 1990). Even though *in vitro* procedures have been designed to minimize disruption of the native structure of chromatin, even slight variations in the ionic strength in which the chromatin is isolated can significantly influence the domain size measured [for discussion, see Jackson et al. (1990)]. The nicking and Me_3-psoralen photobinding required for domain measurements with this approach are performed in intact, living cells. Moreover, Me_3-psoralen binding is sufficiently weak that nucleosomes are not displaced [for discussion, see Sinden et al. (1982)].

The size of the topological domain containing the rDNA arrays was analyzed. The ribosomal RNA genes are organized in multiple arrays in several chromosomal locations in a living human cell. Each repeat unit is 44 kb which contains a 13 kb transcribed region with a 31 kb spacer (Henderson et al., 1973; Worton et al., 1988). These rDNA genes are wound with unrestrained supercoiling (Ljungman & Hanawalt, 1992; Jupe et al., 1993) which allows measurement of domain sizes. The range of the measured domain size of 30 000 ± 1500 to 45 000 ± 2000 bp is similar to the rDNA repeat length of 44 kb, and it is conceivable that each repeat is organized into a separate functional domain. This would agree with, but certainly not prove, a model that domains are functional in organization with a transcribing region defining a domain (Jackson et al., 1992).

Different doses of nicking were required to relax all supercoils in the *hph* gene in individual cell lines. Supercoiling in F14C-8 and 122-6 was completely relaxed after 1 min of nicking, as indicated by the very similar $R_{I/N}$ values for the 1 and 2 min nicking treatments (Figure 4, shaded bars). The two *hph* genes in F14C-23 required 2 min of nicking to produce an $R_{I/N}$ value ($R_{I/N} = 1.35$) similar to that for cell lines F14C-8 and 122-6. The results are consistent with different topological domain sizes ranging from <95 000 ± 5000 bp per domain to >150 000 ± 6000 bp per domain. This conclusion requires the assumption that the nicks from BrdUrd photolysis by 313 nm light are introduced at identical rates in the domains containing the *hph* gene in the different cell lines. However, different chromosomal regions containing the *hph* gene may become nicked at different rates due to different chromatin organization in various parts of the genome, such that a more accessible region is more prone to nicking (as well as cross-linking) (Jupe et al., 1993; Ljungman & Hanawalt, 1992). Thus, while we have demonstrated the utility of this approach for measuring topological domain sizes in human cells, an accurate measure of the domain sizes for each *hph*-containing locus will require measurement of nicks within the *hph* gene and flanking DNA sequence.

The fit of the $R_{I/N}$ values to the theoretical curves is not as good as that observed for relaxation of the *E. coli* (Sinden & Pettijohn, 1981) or bacteriophage T4 (Sinden & Pettijohn, 1984) chromosomes. The nonfit is consistent with a model in which all domains do not have a single average domain size (m). The data would be consistent with a model in which there are two or more populations of different domain sizes, as may be the case for the *hph*-containing domain in cell lines F14C-8 and 122-6.

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