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Presence of negative torsional tension in the promoter region of the transcriptionally poised dihydrofolate reductase gene *in vivo*

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ABSTRACT

DNA topology has been suggested to play an important role in the process of transcription. Negative torsional tension has been shown to stimulate both pre-initiation complex formation and promoter clearance on plasmid DNA *in vitro*. We recently showed that genomic DNA in human cells contains localized torsional tension. In the present study we have further characterized and mapped torsional tension in the dihydrofolate reductase (*DHFR*) gene in Chinese hamster ovary (CHO) cells and investigated the effects of differential rates of transcription on the magnitude and location of this tension. Using psoralen photocross-linking in conjunction with X-irradiation, we found that relaxable psoralen hypersensitivity was specifically localized to the promoter region of the serum-regulated *DHFR* gene in serum-stimulated, but not in serum-starved, cells. Moreover, this hypersensitivity did not appear to be caused by transcription elongation, since it persisted in cells in which transcription of the *DHFR* gene had been reduced by the transcription inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). We suggest that the generation of negative torsional tension in DNA may play an important role in gene regulation by poising genes for transcription.

INTRODUCTION

The mechanism of transcription regulation in eukaryotic cells is not yet completely understood. RNA polymerases require multiple transcription factors to acquire promoter specificity, as well as a large number of auxiliary proteins involved in transcriptional activation (1). In addition, it has been suggested that the superhelical topology of DNA plays an important role in the process of transcription and in the maintenance of active chromatin. Studies have shown that induction of strand breaks in cellular DNA will lead to the disappearance of DNAse I hypersensitive sites in active genes (2,3) and loss of transcription (4–6).

A number of studies using plasmid DNA templates have implicated a role for torsional tension in the early stages of transcription (for reviews see 7,8). Negative torsional tension has been shown to stimulate transcription on plasmid DNA both *in vitro* (9–13) and *in vivo* (14–19). It has been suggested that assembly of the pre-initiation complex, which is the rate limiting step in transcription initiation, is stimulated when the template is under negative superhelical tension (20). It has also been shown that promoter clearance, which is the transition from the initiation complex to the elongation complex, is stimulated *in vitro* by negative supercoiling (12). Positive torsional tension, on the other hand, has been found to be inhibitory to transcription (21).

The measurement of torsional tension in genomic DNA, in contrast to plasmid DNA, has been hampered by technical limitations. However, a promising approach was developed by Sinden and co-workers utilizing photoactivated psoralen in conjunction with X-irradiation to detect torsional tension in genomic DNA (22,23). The basis of this technique is that photoactivated psoralen binds preferentially to DNA that is underwound by negative torsional tension (22,24). By comparing the amount of DNA-bound psoralen formed in cells previously X-irradiated (to relax any tension) with the amount formed in unirradiated cells, the presence of torsional tension can be determined (23). Using this technique, Sinden and co-workers found that, in contrast to bacterial cells, no net torsional tension was detected in the bulk DNA of insect and human cells (22). However, since the psoralen binding assay measures an average tension over the whole genome, it would probably fail to detect any localized tension.

Based on the principles of the psoralen approach of Sinden and co-workers, we recently developed a technique to measure torsional tension in specific sequences of genomic DNA (25). This technique involves the alkali fragmentation–renaturation–hydroxylapatite (AFRHA) assay (26,27) coupled to slotblot hybridization detection of specific sequences. Using this technique, we recently showed that the DNA in the 5′-end of the expressed dihydrofolate reductase (*DHFR*) gene and in the ribosomal transcriptional unit in growing human cells is under negative torsional tension, while the 3′-end of the *DHFR* gene contains slightly positively twisted DNA. With a similar psoralen

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photocross-linking approach, Jupe and co-workers have shown that the heat shock gene *hsp70* in *Drosophila* cells contains negative torsional tension (28).

In the present study we set out to further characterize and map the torsional tension in the *DHFR* gene using the AFRHA/slotblot technique. Our aim was to more precisely determine the localization and magnitude of the negative torsional tension and to study whether the rate of transcription of the *DHFR* gene would affect the magnitude and location of this tension. The level of photocross-linking induced in six regions within the *DHFR* gene domain was examined in CHO B11 cells and compared with the level of photocross-linking formed in relaxed DNA in X-irradiated cells. The results indicate that the promoter region of the serum-regulated *DHFR* gene becomes psoralen hypersensitive, in comparison with the X-irradiated cell sample, in response to serum stimulation. Furthermore, this hypersensitivity did not appear to be due to transcription elongation-induced supercoiling. We propose that the introduction of negative torsional tension in the *DHFR* gene in response to serum stimulation is part of a process to poise the *DHFR* gene for transcription.

MATERIALS AND METHODS

Cell culture

The methotrexate-resistant Chinese hamster cell line CHO K1 B11 [0.5] containing a 50-fold amplification of the *DHFR* gene (29) was plated at a density of 1 500 000 cells/60 cm^2 culture dish in minimal essential medium (MEM) supplied with 10% dialyzed fetal bovine serum, 2 mM glutamine, 1 × non-essential amino acids, 100 IU penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin and 5000 Bq/ml [methyl-^{3}H]thymidine or 150 Bq/ml [methyl-^{14}C]thymidine (Amersham). Methotrexate (Calbiochem Corp., La Jolla, CA) was added to the medium to a final concentration of 0.55 μM to select for cells maintaining the amplified *DHFR* gene. The medium was removed 24 h after seeding the cells, followed by a rinse with phosphate-buffered saline (PBS) and the addition of a non-radioactive medium containing 0.1% fetal bovine serum. After 4 days incubation in the low serum medium (serum starvation), the cells were stimulated by exchanging the medium for a medium containing 15% fetal bovine serum. The experiments were performed 15 h after serum addition to allow for maximal transcription of the *DHFR* gene (30).

GM38 normal, non-fetal human skin fibroblasts, passages 14–20, were grown to confluence on 24-well culture plates (~200 000 cells/well) in Dulbecco's modified Eagle's medium supplied with 15% fetal calf serum, antibiotics (90 IU/ml penicillin and 90 μg/ml streptomycin) and 500 Bq/ml [methyl-^{14}C]thymidine (Amersham).

X-irradiation

Culture dishes with lids removed were irradiated on ice in 1 ml PBS solution. The culture dishes were rotated in the X-ray machine to ensure equal exposure. It should be noted that in previous studies (25) irradiations were performed in 10 ml PBS with the culture dish lid on. This was later found to reduce the dose actually reaching the cells by a factor of about two, due to the low energy of the lithium X-ray machine used. Thus the X-ray doses reported in our previous study (25) should by divided by a factor of two to obtain the actual dose the cells were exposed to.

Measurement of psoralen-induced DNA cross-links in nucleoid DNA by AFRHA

Nucleoid monolayers were prepared from confluent monolayers of GM38 human fibroblasts as previously described (27,31), with 0.8 mM $MgCl_2$ included in the permeabilization/2 M salt solution (32). The choice of human fibroblasts (GM38) as the source for the preparation of nucleoid monolayers was determined by the fact that CHO B11 cells detach following permeabilization and salt treatment, while GM38 fibroblasts do not and form nucleoid monolayers. These nucleoid monolayers were incubated with 0.5 μg/ml PBS and 4′-hydroxymethyl-4,5′,8-trimethylpsoralen (HMT) for 5 min on ice in the dark followed by exposure to 18 kJ/m^2 365 nm (UVA) light at 4°C. The overall frequency of cross-links induced in nucleoid DNA was measured using the AFRHA technique (26,27). X-irradiated and unirradiated cells or nucleoids were treated with HMT and UVA followed by lysis and DNA fragmentation in alkali. After neutralization, the cross-linked fragments were allowed to renature, after which single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) were separated using hydroxylapatite chromatography and counted by liquid scintillation. As radiation-induced breaks appear in the fragments containing cross-links, they will lose DNA material when denatured in alkali and thus shift the ratio of dsDNA/ssDNA. The DNA cross-linking values were therefore corrected for the loss of ^{14}C signal from the cross-linked DNA fraction as a result of X-ray-induced strand breaks. These correction factors were calculated from a control experiment in which psoralen cross-linked samples were irradiated with increasing doses of X-rays and the losses of ^{14}C signal from the dsDNA fractions were determined (data not shown).

In vivo transcription

Cells previously labeled with [^{14}C]thymidine were incubated for 30 min at 37°C in 1 ml labeling solution consisting of 800 μl conditioned medium [high serum, low serum or high serum with 100 μM [5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB)] mixed with 200 μl aqueous solution containing 200 μCi (7.4 MBq) [5-^{3}H]uridine (Amersham). After 30 min RNA labeling, the cells were put on ice and washed several times with ice-cold PBS. Total RNA was extracted essentially as previously described (33), then partially hydrolyzed in cold alkali to produce RNA fragments in the size range 200 bases (34). These RNA fragments were hybridized at 65°C for 48 h to 2–4 μg denatured and sonicated DNA probes immobilized on Hybond N+ membranes (Amersham). Following a stringency wash at 65°C in 0.1× SSPE and 0.1% SDS for 60 min, the membranes were dried and the disintegration of ^{3}H was counted in a scintillation counter. The values were corrected for relative DNA content (measured from the ^{14}C count from an aliquot of sample following the DNase I digestion step in the RNA extraction protocol; 33).

In vitro nuclear run-on transcription

The nuclear run-on procedure was performed as previously described (34,35). In short, nuclei were isolated from ~10^7 [methyl-^{3}H]thymidine-labeled cells and heterogeneous nuclear RNA was labeled *in vitro* with ^{32}P-labeled UTP for 15 min at 30°C. RNA was then isolated, partially hydrolyzed in cold alkali (34) and hybridized to 2–4 μg DNA probes immobilized on Hybond N+ membranes. Membranes were stringency washed at

65°C in 0.1× SSPE and 0.1% SDS for 60 min. Autoradiographs of different exposures were scanned using a scanning densitometer (Helena Laboratories) and peak values were corrected for cell input (^{3}H count of isolated nuclei prior to the run-on protocol).

Measurement of DNA cross-links in specific sequences using the AFRHA/slotblot technique

The photocross-linking of cellular DNA with HMT (1 μg/ml PBS) and UVA irradiation (18 kJ/m^2), as well as the resolution of these cross-links in specific DNA sequences using the AFRHA/slotblot technique, were performed as previously described (25), with a few modifications. These modifications included (i) a sonication for 30 s at output 3 instead of 1.5 using a Branson sonifier and (ii), following the sonication, samples were heated at 55°C for 10 min before being loaded onto the hydroxylapatite columns at a temperature of 70°C. These modifications were performed to lower the renaturation rate of non-cross-linked DNA (control).

All samples were loaded by volume in triplicate onto Hybond N+ nylon membranes using a slotblot apparatus (Schleicher & Schuell). Dilutions were made of all samples so that not more than 100 ng DNA, as measured by Hoechst fluorescence, was loaded into any one slot. Hybridization with ^{32}P-labeled, nick-translated DNA probes and washing of the membranes was performed as previously described (25). However, membranes hybridized with probe 1 (pZH24) were submitted to a stringency wash at 70°C, instead of 60°C (which was used for all other probes), to reduce non-specific hybridization, as determined by hybridization to a Southern transfer of restricted DNA (data not shown). X-ray films (Kodak) were exposed to the membranes for 1–20 h in the absence of intensifying screens. The resulting autoradiographs were scanned and the peak height values of the ssDNA and dsDNA bands were used to calculate the number of cross-links (C-L) per fragment (about 2 kb in average size) according to the formula:

$$\text{C-L/fragment} = -(\ln F_{ss} - \ln F_{ss\ background})$$

where F_{ss} = ssDNA/(ssDNA + dsDNA) and $F_{ss\ background}$ is the fraction of DNA that renatured in control cells not treated with HMT and UVA (control). The mean cross-linking value from the triplicate blotted samples was used to evaluate whether X-irradiation prior to the HMT and UVA treatments had an effect on the level of DNA cross-links. To compensate for variations in psoralen photocross-linking caused by factors other than torsional tension, we used the ratios of photocross-linking in total DNA as internal controls to normalize the ratios obtained with the different DNA probes. This was done since the photocross-linking of total genomic DNA did not show a significant effect of prior X-irradiation.

RESULTS

Photoactivated psoralen and X-rays to determine the presence of torsional tension

The psoralen derivative HMT was used in this study as a probe for DNA torsional tension. HMT readily enters intact cells and intercalates into DNA. Upon photoactivation with 365 nm light (UVA), DNA interstrand cross-links are formed (36). It has been shown that the intercalation of psoralen into DNA is favored if the DNA is under negative superhelical tension (22,23). Thus more HMT-induced DNA cross-links are expected in negatively supercoiled DNA compared with relaxed DNA.

To show that HMT-induced DNA cross-links are formed preferentially in negatively supercoiled DNA as compared with relaxed DNA, we irradiated nucleoid monolayers with increasing doses of X-rays prior to the HMT and UVA treatment. Nucleoid monolayers are the leftovers from a detergent and high salt treatment of cellular monolayers. Nucleoids contain histone-depleted DNA anchored to the nuclear matrix, which is part of a residual cellular structure attached to the culture dishes (27,31). The procedure to produce nucleiods induces no measurable DNA strand breaks (31) and the DNA superhelical density becomes about –0.06 as the nucleosomal histones are removed (7). The frequency of cross-links induced in nucleoid DNA was measured using the AFRHA technique (26,27). As can be seen in Figure 1A, increased DNA nicking by X-rays resulted in decreasing numbers of DNA cross-links formed in the nucleoid DNA. Thus the psoralen DNA cross-links were formed more efficiently in intact, supercoiled nucleoid DNA than in nicked DNA. Assuming a Poisson distribution of the radiation-induced DNA strand breaks, we estimate the D_0 (dose at which 37% of targets are still intact) to be ~0.75 Gy. This dose results in about one DNA strand break/60 kb nucleoid DNA (31). Thus the average target size for relaxation of superhelical tension in nucleoid DNA is ~60 kb.

The experiment was also performed by irradiating intact cells with increasing doses of X-rays prior to preparing the nucleoids. The result is essentially the same (Fig. 1B), except that significantly higher X-ray doses were required for relaxation, due to the protective effect of chromatin proteins (31,37). In this case D_0 is estimated to be ~25 Gy, which is equivalent to about one strand break/190 kb cellular DNA (32).

Taken together, these experiments show that the use of psoralen photocross-linking in combination with X-rays as a nicking agent is a valid approach for the examination of superhelical tension in DNA. Intact nucleoids, having a superhelical density of about –0.06 (7), accumulated 30–50% more DNA cross-links than relaxed nucleoids. We also conclude that nucleoid DNA is organized into independent topological domains with an average size of ~60 kb, which is consistent with other studies (38). Interestingly, when X-irradiation was performed on intact cells prior to the 2 M NaCl treatment the target for relaxation was estimated to be about three times larger. This inconsistency in the results could perhaps be due to additional attachments to the nuclear matrix of exposed DNA sequences in the histone-free nucleoids, leading to the formation of smaller domains. Finally, in Figure 1B it can be seen that a dose of 75 Gy is sufficient to completely relax chromatin domains in intact cells and therefore this dose was chosen in subsequent experiments to relax potential torsional tension in intact cells.

Analysis of transcriptional activity in the *DHFR* gene domain

In order to correlate the topological status of specific DNA sequences in the *DHFR* gene domain (Fig. 2) with transcriptional activity, we first analyzed the rate of RNA synthesis in cells subjected to different growth conditions and the transcription inhibitor DRB. DRB has in some studies been suggested to inhibit transcription at the level of initiation (39–42), while other studies have shown that elongation and not initiation appears to be the target for DRB (43,44). The mechanism of inhibition may be related to its ability to interfere with the activities of casein kinase II (45) and CTD kinase (46). DRB was chosen as a RNA

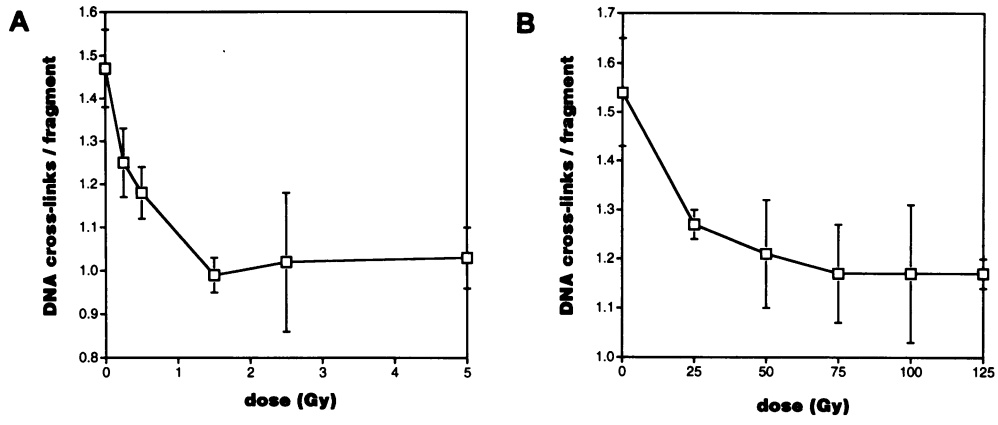

Figure 1. The number of DNA cross-links induced by photoactivation of HMT in nucleoid DNA as a function of X-ray dose given prior to the photocross-linking. The number of interstrand cross-links per DNA fragment was determined by the AFRHA technique (see Materials and Methods). The values represent the mean of four different biological samples with bars showing the sample standard deviation. (**A**) Cells were permeabilized with Triton X-100 and treated with 2 M salt to form nucleoid monolayers. These monolayers were exposed to increasing doses of X-rays followed by cross-linking with HMT (0.5 μg/ml) and UVA (18 kJ/m^2). (**B**) Cells were first irradiated with X-rays, then treated with Triton X-100 and 2 M salt to form nucleoid monolayers followed by photocross-linking with HMT (0.5 μg/ml) and UVA (18 kJ/m^2).

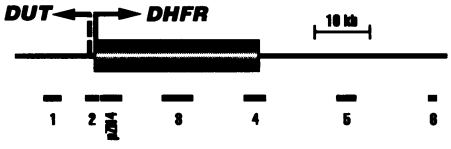

Figure 2. Map of the *DHFR* gene and *DUT* showing the locations of the DNA probes used. The probes are: 1, a 1.74 kb *Eco*RI–*Hin*dIII fragment termed pZH24 (a gift from G. Spivak); 2, a 1.33 kb *Eco*RI fragment –935 to + 395 relative to the *DHFR* start codon termed ME13.3 (71); 3, a 5.3 kb *Bam*HI fragment termed pB6-14 (74); 4, a 2.1 kb *Bam*HI fragment termed pB13-7 (74); 5, a 1.7 kb *Eco*RI fragment termed pZH26 (75); 6, a 0.3 kb *Bam*HI–*Xba*I fragment termed pZH16 (a gift from J. Hamlin); pZH4, a 1.9 kb *Eco*RI–*Bgl*II fragment starting at about +700 and covering most of intron 2 and exon 3 (76) and pZH8 (not shown); a 4.8 kb *Sal*I–*Eco*RI fragment covering most of the 28s rRNA gene of mouse (a gift from G. Spivak and I. Mellon).

polymerase II inhibitor because, in contrast to α-amanitin, it readily enters living cells (39,47).

Using an *in vivo* [^{3}H]uridine labeling approach, we found that synthesis of RNA from the *DHFR* gene was ~10-fold lower in serum-starved cells compared with serum-stimulated cells and almost completely inhibited in DRB-treated cells (Fig. 3A). Results using probe 2, which is complementary to transcripts produced from the first 450 bases of the *DHFR* major initiation site and the first 700 bases from the divergent upstream transcript (*DUT*) major initiation site, show no indications of promoter-proximal, DRB-resistant synthesis of stable RNA. However, we cannot rule out the possibility that some initiation may occur in the presence of DRB, generating prematurely terminated RNA transcripts. If this is the case, the short transcripts produced must be rapidly degraded during the labeling period to evade detection in our hybridization experiments.

To further analyze the rate of RNA synthesis, we performed nuclear run-ons to measure *in vitro* elongation of transcripts initiated *in vivo*. The amount of [^{32}P]UTP incorporated into *DHFR* transcripts was found to be 2–4-fold lower in serum-starved cells compared with serum-stimulated cells (Fig. 3B). In cells treated with 100 μM DRB during the last hour of the 15 h serum stimulation, the amount of *in vitro* labeling of nascent nuclear RNA was reduced to ~35% in promoter-proximal regions of the *DHFR* and *DUT* genes and to 5–15% downstream in the *DHFR* gene.

We conclude from the results obtained *in vivo* and *in vitro* that serum starvation lowered the rate of transcription throughout the *DHFR* gene, while DRB was found to reduce the synthesis of *DHFR* RNA even further. The inhibition by DRB appeared to hold true even for promoter-proximal regions, a result which is in agreement with findings *in vivo* for the β-hemoglobin gene in Friend erythroleukemic cells (42), but in contrast to the heat shock genes in *Drosophila* cells (44), the c-*myc* gene injected into *Xenopus* oocytes (48) and virus-encoded genes in infected mammalian cells (49,50). It is possible that DRB affects transcription in a gene-specific manner, where the particular constitution of promoter sequences together with their bound *trans*-acting factors dictates the outcome of the DRB treatment.

Relaxable psoralen hypersensitivity is specifically localized to the promoter region

We examined the presence of torsional tension at six different locations in the *DHFR* gene domain in serum-stimulated CHO B11 cells using psoralen photocross-linking measured by the AFRHA/slotblot technique (25). Following the AFRHA procedure, the ssDNA and dsDNA fractions were immobilized onto nylon membranes and subsequently hybridized to DNA probes. The number of cross-links per DNA fragment was obtained from the ratio of dsDNA to ssDNA (see Materials and Methods). If the number of cross-links in a specific sequence was lowered by prior X-irradiation, the sequence was assigned as under negative torsional tension.

Comparing the ratios of dsDNA to ssDNA in cells X-irradiated prior to photocross-linking (X-HMT) with the ratios obtained in cells irradiated after (HMT-X), we found that the only region where prior X-irradiation altered the amount of photocross-linking (dsDNA) was in the region detected by probe 2, which hybridizes to the promoter DNA of the *DHFR* gene. The effect of prior X-irradiation on photocross-linking of the *DHFR* promoter region was not as pronounced in CHO cells as it was in

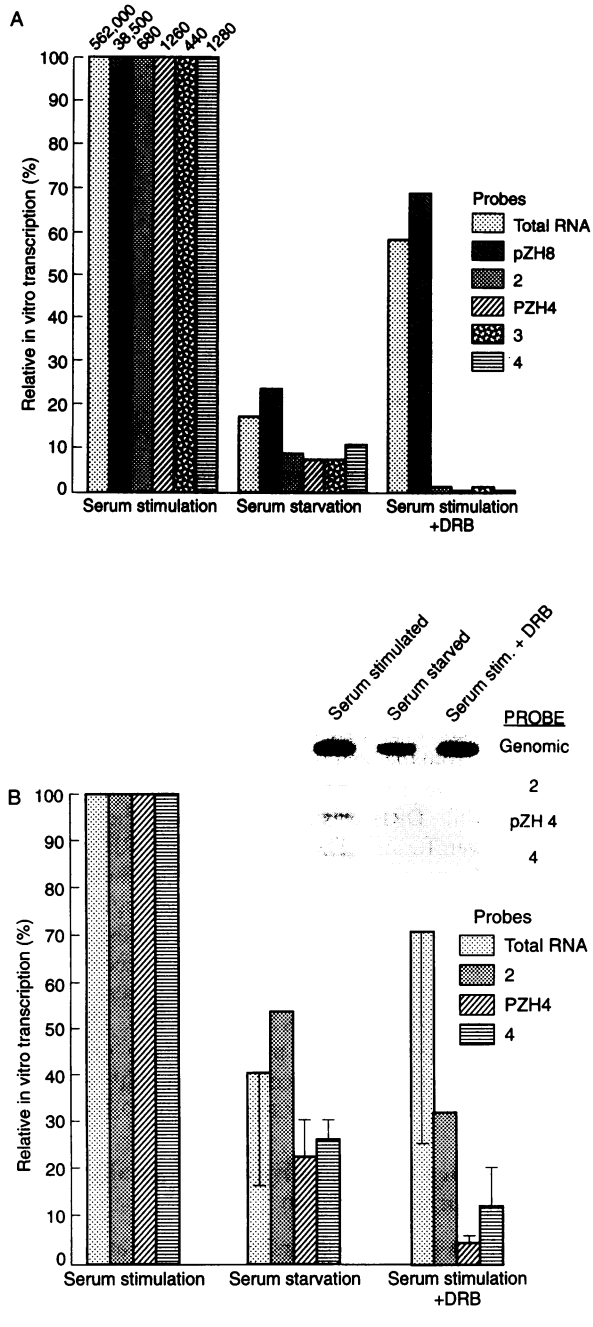

Figure 3. (**A**) *In vivo* transcriptional analysis of the *DHFR* gene in serum-stimulated cells, serum-starved cells and in serum-stimulated cells treated with 100 μM DRB for 60 min prior to harvest. The numbers shown for the serum-stimulated cells represent the ^{3}H counts (DNA input corrected c.p.m.) from the hybridized RNA. All other values are expressed relative to these counts and represent the mean of three independent biological samples, with bars showing the sample standard deviation. The relative values of total RNA were obtained by counting a small sample of the isolated RNA directly in a scintillation counter. The c.p.m. values for the DRB-treated cells were multiplied by a factor of two to correct for the lower uptake of [^{3}H]uridine in the presence of DRB (77,78). The probe pZH8 is complementary to 28s rRNA transcripts synthesized by RNA polymerase I. (**B**) *In vitro* nuclear run-on analysis of transcription initiated *in vivo*. Insert: an autoradiograph from a typical nuclear run-on experiment. The rates of nascent RNA synthesis are expressed relative to the level of RNA synthesis occurring in the nuclei from serum-stimulated cells. The values were corrected for DNA input and represent the mean of one to three experiments, with bars showing the sample standard deviation.

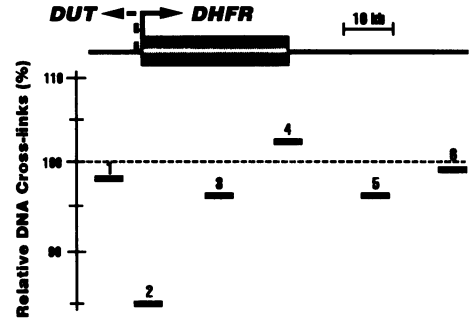

Figure 4. The level of DNA cross-links induced by HMT and UVA in X-irradiated cells (X-HMT) relative to unirradiated cells (HMT-X). The black bars represent the mean values of 16–17 biological experiments with standard deviations shown in Table 1. The values obtained for the different probes are positioned to show their location within the *DHFR* gene domain. Values below 100% indicate that X-irradiation prior to the photocross-linking reaction lowered the yield of DNA cross-links and were taken to mean that the DNA sequence is under negative torsional tension. The promoter region was found to contain negative torsional tension ($P < 0.001$).

human cells (25). In order to statistically verify the existence of negative torsional tension in the promoter region, we performed >15 separate biological experiments; the average values are presented in Figure 4 and in Table 1. We conclude that the DNA in the *DHFR* promoter region is under negative superhelical tension in serum-stimulated cells (significance $P < 0.001$), while in contrast, sequences upstream, downstream, in the middle and at the 3′-end of the transcriptional unit do not appear to be under any significant tension.

Table 1. Photocross-linking levels of X-irradiated cells relative to unirradiated cells

Probe[a]	Serum stimulation n = 16–17	Serum starvation n = 3–5	Serum stimulation + DRB n = 5	Serum stimulation + mimosine n = 5–6
1	97 ± 8[b]	103 ± 11	105 ± 7	nd[d]
2	85 ± 7*[c]	108 ± 14	87 ± 4*	87 ± 6*
3	96 ± 10	100 ± 10	107 ± 6	nd
4	102 ± 10	104 ± 6	105 ± 5	109 ± 7
5	96 ± 6	109 ± 10	100 ± 3	nd
6	99 ± 16	102 ± 5	98 ± 6	110 ± 16

The values represent the average of n biological experiments and are expressed as a percentage of cross-links induced in unirradiated cells.
[a]Nick-translated DNA probes described in Figure 2.
[b]Average value of n biological experiments showing sample standard deviation.
[c]Asterisk indicates values significantly different from 100% using Student's t-test with $P < 0.001$, 0.002 and 0.01 for serum stimulated, serum stimulated + DRB and serum stimulated + mimosine, respectively.
[d]nd, not determined.

To estimate the magnitude of superhelical density in the DNA of the *DHFR* promoter, we extrapolated values from the photocross-linking results obtained from supercoiled and relaxed nucleoids. Intact nucleoid DNA, having a superhelical density of about –0.06 (7), was found to be cross-linked 30–50% more

efficiently by psoralen and UVA than relaxed nucleiods (see Fig. 1). It then follows that the 15% higher cross-linking level found in the *DHFR* promoter in CHO cells, compared with X-irradiated cells, extrapolates to a superhelical density of –0.02 to –0.03. This value is lower than –0.06, which we estimate to be present in the *DHFR* promoter in growing human cells (25). The validation of these extrapolations is of course contingent on the rationale of comparing photocross-linking data from nucleoids and intact cells.

Torsional tension in the promoter region is dependent on serum stimulation, but not on transcription elongation

Next we wanted to address the question of whether or not the tension observed in the promoter region of the *DHFR* gene was the direct result of transcription elongation (51). We investigated the topological state within the *DHFR* gene domain in serum-starved and in DRB-treated cells and compared these results with the results obtained from the serum-stimulated cells. Despite the fact that the *DHFR* gene is transcribed even under serum starvation conditions, although at a lower rate (see Fig. 3), we found no evidence for torsional tension in any of the six DNA regions studied (Table 1). However, in serum-stimulated cells treated with the transcriptional inhibitor DRB, in which *DHFR* transcription was found to be lower than in serum-starved cells, the negative torsional tension was retained (Table 1). These results suggest that the negative torsional tension detected in the promoter region of the *DHFR* gene is not merely the result of transcription-induced supercoiling. We also ruled out the possibility that DNA replication was responsible for the appearance of negative torsional tension in the promoter region, since the tension was still present even after suppression of replication with mimosine (Table 1). We conclude that the formation of torsional tension in the promoter of the *DHFR* gene is not dependent on transcription elongation or DNA replication, rather, it is dependent on a process induced by serum stimulation.

DISCUSSION

In a previous study using the AFRHA/slotblot assay we found evidence for localized torsional tension in the *DHFR* gene in living human cells (25). In the present study we have further characterized and mapped the torsional tension in the *DHFR* gene domain in CHO B11 cells, estimated its superhelical density and studied the effects of differential transcription on this tension. In addition, we evaluated the validity of using the psoralen photocross-linking approach to study DNA torsional tension by testing it on nucleoid DNA.

The test of the psoralen photocross-linking assay, using nucleoid DNA as a supercoiled substrate, showed that as nucleoid DNA was nicked with increasing doses of X-rays prior to psoralen treatment, the number of DNA cross-links formed decreased to a certain level at which further X-irradiation had no effect. Our results suggest that one nick per DNA loop or domain is sufficient to eliminate psoralen hypersensitivity. In addition, we conclude from our *in vivo* results that X-irradiation does not cause a general effect on psoralen photocross-linking, a finding which is supported by others (28). Thus we believe that the approach of using psoralen photocross-linking in combination with X-rays as a nicking agent is valid and useful for the examination of DNA topology *in vivo*. However, the true nature of the tension-dependent DNA structure that is detected as psoralen hypersensitive sites is not known.

Using CHO B11 cells, we found that negative superhelical tension is specifically localized to the promoter region of the *DHFR* gene in serum-stimulated cells (Fig. 4). This finding is in agreement with our previous study using growing human cells (25). Extrapolating from the *in vitro* photocross-linking of nucleoids, we estimate that the DNA in the *DHFR* promoter has a superhelical density of about –0.02 to –0.03, while the corresponding value from the human *DHFR* promoter is –0.06. It is possible that the superhelical density is locally considerably higher, but our psoralen photocross-linking assay would not be able to give any information on heterogeneous binding within the 2 kb sized regions measured. However, even at a density as low as –0.02 it has been shown *in vitro* that assembly of the pre-initiation complex on the promoters of many genes is stimulated (52). Thus although the magnitude of the cross-linking changes are small in our assay, the average tension we estimate to be present in the *DHFR* promoter region *in vivo* should be sufficient to stimulate pre-initiation complex assembly and thereby stimulate the rate of transcription (11,16).

It has been shown that as a gene is being transcribed, positive supercoils accumulate in the DNA template in front of the RNA polymerase, while negative supercoils accumulate behind it (51,53–57). Although transcription-induced supercoiling is thought to be relieved by DNA topoisomerase I (58,59), it is possible that some supercoils 'escape' and may accumulate in the promoter region between two divergently transcribed genes. This hypothesis was tested by examining the tension in the *DHFR* promoter under conditions in which the rate of transcription was modified by serum starvation or the transcription inhibitor DRB. Our results suggest that the presence of tension in the promoter does not correlate with the rate of DHFR RNA synthesis. Negative torsional tension was found to be present in the *DHFR* promoter even after transcription of both the *DHFR* gene and the divergent *DUT* gene had been severely inhibited by DRB. On the other hand, no tension was detected in the *DHFR* promoter in serum-starved cells, although the level of *DHFR* transcription in serum-starved cells was found to be higher than in DRB-treated cells. We cannot totally exclude the possibility that some initiation of the *DHFR* and *DUT* genes may take place in the presence of DRB, leading to transcription-induced supercoiling. In addition, we ruled out that the tension was caused by DNA replication, since the same level of tension was found in cells incubated with or without the replication inhibitor mimosine (see Table 1). We conclude that the negative torsional tension detected in the promoter region of the *DHFR* gene is induced by a process triggered by serum stimulation and does not appear to be the result of transcription elongation. This finding supports studies in *Xenopus* oocytes, which have shown that negative supercoiling can be introduced into plasmid DNA independently of ongoing transcription (60).

If the observed tension in the *DHFR* promoter is not caused by elongating RNA polymerases, how is it induced? Superhelical tension can potentially be introduced into chromosomal DNA by the removal or modification of nucleosomes (7,8,61–63) or perhaps by the action of an enzyme complex involving topoisomerase II (64,65). An intriguing candidate responsible for the induction of negative supercoiling in the *DHFR* gene in serum-stimulated cells is the transcription factor E2F, which has recently been recognized as being a key component in activation

of the *DHFR* gene at the G1/S border (66,67). It is possible that the E2F complex, perhaps in conjunction with other cellular components (68,69), modifies the DNA topology in the *DHFR* domain in response to serum stimulation.

How can superhelical tension be localized to a region that is smaller than the gene domain itself? One possible explanation for our finding that negative torsional tension was exclusively localized to the promoter region of the *DHFR* gene could be that this region is organized into a small sub-domain in which the DNA is specifically maintained under negative torsional tension. The caveat, however, is that X-ray-induced strand breaks spaced at an average of >50 kb apart were able to completely relax the tension in the DNA of this promoter region (see 25 and Materials and Methods). It is thus difficult to support a model in which the promoter DNA is organized into a sub-domain that on the one hand can keep torsional tension enclosed, but on the other hand cannot withstand the relaxation caused by strand breaks induced outside the boundaries of this sub-domain. An alternative model is that negative torsional tension may be propagated throughout the gene domain, but that the promoter DNA acts as a 'sink' for most of this tension, forming a psoralen hypersensitive DNA structure. A DNA strand break anywhere in the gene domain will relieve the tension and reverse the psoralen hypersensitivity of the promoter region. It is conceivable that the specific features of the *DHFR* promoter region, such as the primary DNA sequence, the DNA methylation pattern and the chromatin structure (70–72), are responsible for making this region particularly responsive to the effects of superhelical tension. In fact, some promoter regions have been found to contain DNA sequences that preferentially unwind when put under superhelical tension (73). Our observations support such a model in which torsional tension may exert its effects on DNA structure non-randomly in a gene domain, with a preference for promoter sequences.

In conclusion, we have found that the DNA of the serum-regulated *DHFR* gene in CHO cells develops negative superhelical tension in response to serum stimulation. This tension, which does not appear to be induced by elongation by RNA polymerases, becomes specifically localized to the *DHFR* promoter. We propose that unique features of the promoter region predispose the promoter DNA to undergo a structural change, perhaps unwinding, in the presence of negative superhelical tension. This will lead to the poising of the gene for transcription. The ability to specifically localize superhelical free energy to regulatory regions has powerful implications. First, unwinding of the promoter is accomplished by a relatively low input of free energy, since the whole domain need not be supercoiled to a certain density. Secondly, the promoter elements will respond to negative superhelicity introduced anywhere in the gene domain. This structure–function relationship between superhelical tension and transcription initiation may prove to be a general mechanism for the regulation of expression of many genes.

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REFERENCES

1 Tjian,R. and Maniatis,T. (1994) *Cell*, **77**, 5–8.
2 Villeponteau,B., Lundell,M. and Martinson,H. (1984) *Cell*, **39**, 469–478.
3 Villeponteau,B. and Martinson,H.G. (1987) *Mol. Cell. Biol.*, **7**, 1917–1924.
4 Luchnik,A.N., Hisamutdinov,T.A. and Georgiev,P.A. (1988) *Nucleic Acids Res.*, **16**, 5175–5190.
5 Rodi,C. and Sauerbier,W. (1989) *J. Cell Physiol.*, **141**, 346–352.
6 Ghosh,R., Amstad,P. and Cerutti,P. (1993) *Mol. Cell. Biol.*, **13**, 6992–6999.
7 Esposito,F. and Sinden,R.R. (1988) *Oxford Surv. Eukaryote Genes*, **5**, 1–50.
8 Freeman,L.A. and Garrard,W.T. (1992) *Crit. Rev. Eukaryote Gene Expression*, **2**, 165–209.
9 Hirose,S. and Suzuki,Y. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 718–722.
10 Sekiguchi,J.M., Swank,R.A. and Kmiec,E.B. (1989) *Mol. Cell. Biochem.*, **85**, 123–133.
11 Parvin,J.D. and Sharp,P.A. (1993) *Cell*, **73**, 533–540.
12 Goodrich,J.A. and Tjian,R. (1994) *Cell*, **77**, 145–156.
13 Timmers,H. (1994) *EMBO J.*, **13**, 391–399.
14 Harland,R.M., Weintraub,H. and McKnight,S.L. (1983) *Nature*, **302**, 38–43.
15 Weintraub,H., Cheng,P.F. and Conrad,K. (1986) *Cell*, **46**, 115–122.
16 Schultz,M.C., Brill,S.J., Ju,Q.D., Sternglanz,R. and Reeder,R.H. (1992) *Genes Dev.*, **6**, 1332–1341.
17 Chen,D.R., Bowater,R., Dorman,C.J. and Lilley,D. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 8784–8788.
18 Dunaway,M. and Ostrander,E.A. (1993) *Nature*, **361**, 746–748.
19 Bowater,R., Chen,D. and Lilley,D. (1994) *EMBO J.*, **13**, 5647–5655.
20 Mizutani,M., Ohta,T., Watanabe,H., Handa,H. and Hirose,S. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 718–722.
21 Gartenberg,M.R. and Wang,J.C. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 11461–11465.
22 Sinden,R.R., Carlson,J.O. and Pettijohn,D.E. (1980) *Cell*, **21**, 773–783.
23 Sinden,R.R. and Ussery,D.W. (1992) *Methods Enzymol.*, **212**, 319–335.
24 Hyde,J.E. and Hearst,J.E. (1978) *Biochemistry*, **17**, 1251–1257.
25 Ljungman,M. and Hanawalt,P.C. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 6055–6059.
26 Ljungman,M. (1989) *Carcinogenesis*, **10**, 447–451.
27 Ljungman,M. (1990) PhD Thesis, Stockholm University.
28 Jupe,E.R., Sinden,R.R. and Cartwright,I.L. (1993) *EMBO J.*, **12**, 1067–1075.
29 Kaufman,R. and Schimke,R. (1981) *Mol. Cell. Biol.*, **1**, 1069–1076.
30 Feder,J. (1990) PhD Thesis, Stanford University.
31 Ljungman,M. (1991) *Radiat. Res.*, **126**, 58–64.
32 Ljungman,M., Nyberg,S., Nygren,J., Eriksson,M. and Ahnström,G. (1991) *Radiat. Res.*, **127**, 171–176.
33 Sambrook,J., Fritsch,E. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
34 Chen-Kiang,S. and Lavery,D. (1989) *Methods Enzymol.*, **180**, 82–96.
35 Ausubel,F., Brent,R., Kingston,R., Moore,D., Seidman,J., Smith,J. and Struhl,K. (1993) *Current Protocols in Molecular Biology*. John Wiley and Sons, New York, NY.
36 Cimino,G.D., Gamper,H.B., Isaacs,S.T. and Hearst,J.E. (1985) *Annu. Rev. Biochem.*, **54**, 1151–1193.
37 Ljungman,M. and Hanawalt,P.C. (1992) *Mol. Carcinogen.*, **5**, 264–269.
38 Jackson,D., Dickinson,P. and Cook,P. (1990) *EMBO J.*, **9**, 567–571.
39 Egyhazi,E. (1976) *Nature*, **262**, 319–321.
40 Sehgal,P., Darnell,J. and Tamm,I. (1976) *Cell*, **9**, 473–480.
41 Zandomeni,R., Mittelman,B., Bunick,D., Ackerman,S. and Weinmann,R. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 3167–3170.
42 Mukherjee,R. and Molloy,G. (1987) *J. Biol. Chem.*, **262**, 13697–13705.
43 Chodosh,L., Fire,A., Samuels,M. and Sharp,P. (1989) *J. Biol. Chem.*, **264**, 2250–2257.
44 Giardina,C. and Lis,J. (1993) *J. Biol. Chem.*, **268**, 23806–23811.

45 Zandomeni,R., Zandomeni,M., Shugar,D. and Weinmann,R. (1986) *J. Biol. Chem.*, **261**, 3414–3419.
46 Dubois,M.F., Nguyen,V.T., Bellier,S. and Bensaude,O. (1994) *J. Biol. Chem.*, **269**, 13331–13336.
47 Kuwano,M. and Ikehara,Y. (1973) *Exp. Cell. Res.*, **82**, 454–457.
48 Roberts,S. and Bentley,D. (1992) *EMBO J.*, **11**, 1085–1093.
49 Sehgal,P., Fraser,N. and Darnell,J. (1979) *Virology*, **94**, 185–191.
50 Vennström,B., Persson,H., Pettersson,U. and Philipsson,L. (1979) *Nucleic Acids Res.*, **7**, 1405–1418.
51 Liu,L.F. and Wang,J.C. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 7024–7027.
52 Mizutani,M., Ura,K. and Hirose,S. (1991) *Nucleic Acids Res.*, **19**, 2907–2911.
53 Brill,S.J. and Sternglanz,R. (1988) *Cell*, **54**, 403–411.
54 Osborne,B.I. and Guarente,L. (1988) *Genes Dev.*, **2**, 766–772.
55 Giaever,G.N. and Wang,J.C. (1988) *Cell*, **55**, 849–856.
56 Tsao,Y.-P., Wu,H.-Y. and Liu,L.F. (1989) *Cell*, **56**, 111–118.
57 Dröge,P. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 2759–2763.
58 Gilmour,D. and Elgin,S. (1987) *Mol. Cell. Biol.*, **7**, 141–148.
59 Stewart,A. and Schütz,G. (1987) *Cell*, **50**, 1109–1117.
60 Leonard,M.W. and Patient,R.K. (1991) *Mol. Cell. Biol.*, **11**, 6128–6138.
61 Norton,V.G., Imai,B.S., Yau,P. and Bradbury,E.M. (1989) *Cell*, **57**, 449–457.
62 Thomsen,B., Bendixen,C. and Westergaard,O. (1991) *Eur. J. Biochem.*, **201**, 107–111.
63 Lutter,L.C., Judis,L. and Paretti,R.F. (1992) *Mol. Cell. Biol.*, **12**, 5004–5014.
64 Ohta,T. and Hirose,S. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 5307–5311.
65 Ura,K. and Hirose,S. (1991) *Nucleic Acids Res.*, **19**, 6087–6092.
66 Blake,M. and Azizkhan,J. (1989) *Mol. Cell. Biol.*, **9**, 4994–5002.
67 Slansky,J., Li,Y., Kaelin,W. and Farnham,P. (1993) *Mol. Cell. Biol.*, **13**, 1610–1618.
68 Helin,K., Wu,C.-L., Fattaey,A., Lees,J., Dynlacht,B., Ngwu,C. and Harlow,E. (1993) *Genes Dev.*, **7**, 1850–1861.
69 Arroyo,M., Bagchi,S. and Raychaudhuri,P. (1993) *Mol. Cell. Biol.*, **13**, 6537–6546.
70 Shimada,T. and Nienhuis,A. (1985) *J. Biol. Chem.*, **260**, 2468–2474.
71 Mitchell,P., Carothers,A., Han,J., Harding,J., Kas,E., Venolia,L. and Chasin,L. (1986) *Mol. Cell. Biol.*, **6**, 425–440.
72 Barsoum,J. and Varshavsky,A. (1985) *J. Biol. Chem.*, **260**, 7688–7697.
73 Benham,C.J. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 2999–3003.
74 Carothers,A., Urlaub,G., Ellis,N. and Chasin,L. (1983) *Nucleic Acids Res.*, **11**, 1997–2012.
75 Ho,L., Bohr,V.A. and Hanawalt,P. (1989) *Mol. Cell. Biol.*, **9**, 1594–1603.
76 Mellon,I., Spivak,G. and Hanawalt,P.C. (1987) *Cell*, **51**, 241–249.
77 Sehgal,P., Derman,E., Molloy,G., Tamm,I. and Darnell,J. (1976) *Science*, **194**, 431–433.
78 Tamm,I., Hand,R. and Caliguiri,L. (1976) *J. Cell Biol.*, **69**, 229–240.