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Inhibition of transcription in eukaryotic cells by X-irradiation: relation to the loss of topological constraint in closed DNA loops

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ABSTRACT

X irradiation was found to inhibit *in vivo* transcription in mammalian, yeast, insect and avian cells in a dose-dependent manner. Measurements of DNA nicking indicated that about one DNA single-strand break per estimated DNA loop (domain) length is sufficient to explain the effect. The inhibitory effect was partially reversed by post-irradiation incubation of cells. During such incubation DNA nicking was considerably repaired. The size of transcripts was not changed by irradiation. The *in vitro* (run on) activity of RNA polymerase in nuclei isolated from irradiated cells also was not altered. The dose-response curves were different in various cells, correlating with the reported unequal average domain size of supercoiled DNA (and also replicon size) in diverse organisms.

INTRODUCTION

Recently accumulated evidence suggests that topological closure of DNA may be important for transcription in eukaryotes /1-6/. The evidence, however, has been obtained primarily from plasmids or viruses introduced into eukaryotic cells and assembled into chromatin. Although these closed circles of chromatin have been considered to be a good model of a chromatin loop, it is still unclear whether the evidence obtained on viral and plasmid DNA can by applied to understanding of the mechanisms of transcription on chromosomal DNA.

In the present paper, we have investigated the possible relationship between topological closure of DNA in chromatin loops and their transcription using X-irradiation of living cells for transient violation of topological constraint of

DNA. X-irradiation may be a useful approach to this question because it causes rare single-strand (and occasionally double-strand) scisions in DNA while having little effect on other macromolecules within the living cell.

The X-ray-induced killing of cells has no direct relation to our experiments. The lethal effect of X-rays occurs due to improper segregation of chromosomes in metaphase because of chromosome breaks and translocations arising from erroneously repaired DNA double-strand breaks /7-9/. Although the doses of X-rays we employed are higher than the lethal doses required for metaphase death, they are still insufficient to cause interphase death in the majority of cell cultures /8/. Metaphase death, however, is not significant during the time interval of our experiments. Consequently, it does not interfere with the interphase measurements. Moreover, we have measured immediate effects, preceeding most of the DNA repair and related secondary effects.

It was found that X-irradiation reversibly inhibits *in vivo* but not *in vitro* (run on) transcription in mammalian cells at doses inducing approximately one single-strand break per estimated DNA loop length. We suggest that the difference between *in vivo* and *in vitro* results may indicate an altered nucleosome structure (unfolding of nucleosomes) in elastically strained chromatin loops and subsequent refolding of nucleosomes or reconstruction of the inactive ones in a fiber relaxed by X-irradiation. The latter effect is overcome *in vitro* by agents disturbing nucleosome structure (sarcosyl or ammonium sulphate).

MATERIALS AND METHODS

Irradiation and in vivo transcription. Monolayer cell cultures were grown in 1 ml Eagle's medium in 5 ml glass vials with 19 mm flat bottom. ^{14}C-thymidine (10^{-3} mCi/ml) was added 24 h before irradiation to label DNA. Vials were irradiated on RUM-14 apparatus at 2.5 krad/min either on ice or in circulating water at 37°C. Immediately the medium was replaced with 200 µl of warm Eagle's medium containing 0.5 mCi/ml of ^{3}H-uridine and flasks were incubated for 4 min at 37°C. Then cells

were lysed by 1% SDS, 5 mM EDTA, 100 mM NaCl, sonicated at 20 KHz for 30 sec, and the lysate collected on GF/B glass filters which were washed and counted in toluene scintillation fluid. The $^{3}H/^{14}C$ ratio was taken to reflect the efficiency of transcription.

In vitro (run on) transcription. Nuclei were isolated in 0.1 M NaCl, 1 mM EDTA, 0.25% Triton X-100, 10 mM TEA-HCl, pH 7.6, washed with 0.1 M NaCl, 10 mM TEA-HCl, pH 7.6 and were assayed for transcription in 50 mM TEA-HCl, pH 7.6, 6 mM $MgCl_2$, 2 mM $MnCl_2$, 2 mM DTT, 150 mM $(NH_4)_2SO_4$, 50 µg/ml BSA, 0.4 mM each of ATP, CTP, GTP, 25 µCi of ^{3}H-UTP for 30 min at 26°C. In some experiments, sarcosyl was added to 0.4%. The reaction was stopped by the addition of 1% SDS, 5 mM EDTA, 100 mM NaCl; lysates were sonicated, collected on GF/B filters, washed, and radioactivity was counted.

Molecular weight measurements. After cell lysis, 200 µg/ml of proteinase K was added for 2 h or overnight at 37°C, and lysates were layered onto 5-20% sucrose gradients made with 100% formamide, containing 100 mM NaCl and 1 mM EDTA, pH 7.6 (EDTA was added as 0.5 M water solution) /18/. Gradients were spun for 15 h at 18.000 rpm at 20°C. Molecular weights were calculated from mobilities of T4 (170 kb) or T7 (40 kb) phage DNA using the relationship $S1/S2 = (M1/M2)^{0.20}$. The index of 0.20 was found empirically and differed significantly from that of 0.40 derived for water solutions of DNA.

Measurements of transcription of individual gens in Drosophila. Kc cell cultures were concentrated 5-fold by centrifugation and irradiated in Eppendorf tubes kept in circulating water at 25°C. After irradiation ^{3}H-uridine was added to a final concentration of 2.5 mCi/ml and cells were allowed to synthesize RNA at 34-37 C°(in different experiments) to induce heat shock genes and inhibit repair of DNA. Incorporation of isotope was stopped by the addition of 0.2% SDS, 20 mM EDTA. RNA isolated from lysates was treated with 0.1 M NaOH for 15 min to reduce its size and hybridized overnight to DNA probes (bound to nylon Hybond filters) in solution containing 50% formamide, 0,5 M NaCl, 10 mM EDTA, 50 mM Tris-HCl, pH 7.6,

2xDenhart solution without BSA, 0.5% SDS, 500 μg/ml denatured calf thymus DNA.

RESULTS AND DISCUSSION

Reversible inhibition of both hnRNA and ribosomal RNA synthesis after X-irradiation

The dependence of total cellular transcription levels on X-ray dose in CV-1 cells is presented in Fig. 1. It is seen that X-irradiation severely inhibits transcription in a dose-dependent manner. The inhibition was stronger after irradiation at 0°C than at 37°C (compare curves A and B). Cooling of cells is known to inhibit the ligation of DNA nicks induced by radiation. Indeed, we have found that keeping the cells at 37°C after irradiation at 0°C partially restored their transcriptional competence (Fig. 1 curve C) as well as reducing the number of nicks induced by radiation (see below, Fig. 3B). Thus, the difference between the levels of inhibition after irradiation at 0°C and 37°C may be explained by fast repair during irradiation at 37°C. (Control cells and cells receiving a lower dose were kept on ice for the same period as those irradiated for a longer time).

We have to mention here that the induction of DNA single-strand breaks *in vivo* by X-irradiation increased linearly with dose in every studied organism /8/ and it has been indeed the case in our experiments. So, the observed reduction of transcription is proportional to the increase in the number of DNA single strand breaks.

The observed curves obviously are not truly exponential. There are several possible explanations for the non-exponential tails. They may be ascribed to the initiation of transcription from a growing number of DNA nicks at higher dose. Nicks have been shown to be good initiation sites for transcription /10/. Also, the curves may represent the sum of different exponents due to possibly heterogeneous DNA loop sizes. Finally, the tails may be explained by the existence of rare genes which do not switch off after irradiation.

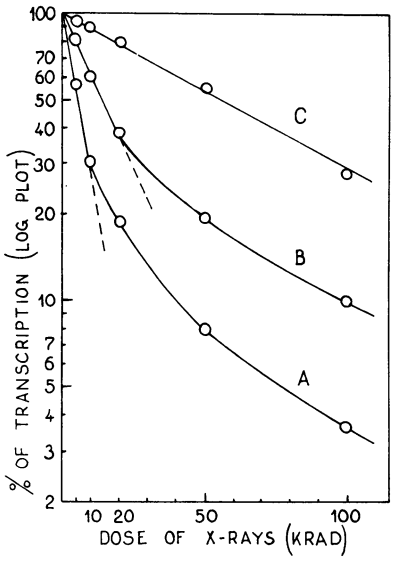

Fig. 1. Inhibition of RNA synthesis in CV-1 cells after X-irradiation. A - irradiation at 0°C; B - irradiation at 37°C; C - irradiation at 0°C with subsequent incubation at 37°C for 2 h before the addition of ^{3}H-uridine.

In the experiments presented in Fig. 1, RNA was labeled for 4 min. With such a short pulse, the label is incorporated mainly into hnRNA. We therefore labeled the cells for 40 min to label mainly ribosomal RNA; as a control 0.05 μg/ml of Actinomycin D was added to some samples to inhibit specifically rRNA synthesis /11/. (The specificity of Actinomycin D inhibition in these cells was checked by RNA electrophoresis). Thus, one can compare the synthesis of rRNA and hnRNA. In both cases, X-rays inhibited transcription almost to the same extent in spite of the fact that the initial level of RNA synthesis after Actinomycin D treatment was lower by 70% (Fig. 2A). Thus, ribosomal and hnRNA synthesis were impared by X-irradiation to a similar extent.

In separate experiments, we determined that the transport and phosphorylation of ^{3}H-uridine were unaffected by X-irradiation. RNA degradation also was not elevated (data not shown).

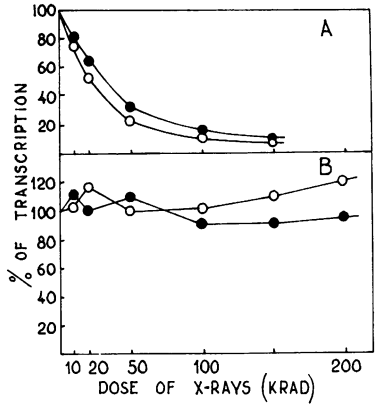

Fig. 2. (A) Inhibition of total (mainly ribosomal) RNA (●—●) or hnRNA (○—○) synthesis after X-irradiation of CV-1 cells. Cells were irradiated at 37°C and then ^{3}H-uridine was added for 40 min. Actinomycin D (0.05 µg/ml) was added before ^{3}H-uridine to assay hnRNA synthesis. (B) Run-on transcription assay on nuclei isolated from CV-1 cells irradiated (at 37°C) at different doses. ○—○ transcription without sarcosyl, ●—● transcription in the presence of 0.4% sarcosyl.

Studies of in vitro (run on) transcription and RNA size measurements

Among possible explanations for the inhibition of transcription could be (1) damage to the transcriptional machinery (2) damage to the DNA template due to nicking, DNA-protein crosslinking or chemical modification of bases by the irradiation, (3) loss of DNA torsional strain leading to the destruction of an active chromatin structure. To choose between these possibilities, we performed the following experiments.

First, we studied run-on transcription in nuclei isolated from irradiated cells. Such transcription represents an elongation of preinitiated RNA chains either in the absence of histones (removed by sarcosyl) or in the presence of severely altered nucleosome structure (in the presence of 0.1-0.4 M ammonium sulphate). It was found that run-on transcription was unaffected by X-irradiation of cells (Fig. 2B). This means that previously initiated RNA polymerase is relatively undamaged, and, moreover, remains bound to template. It also fol-

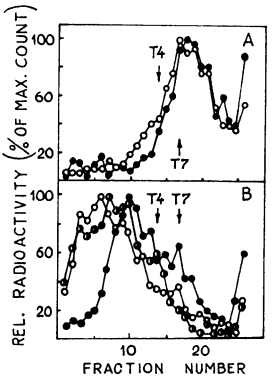

Fig. 3. (A) Determination of size of newly synthesized RNA after X-irradiation of CV-1 cells. (Irradiation at 37°C 4 min pulse of ^{3}H-uridine). O—O RNA from control cells, ●—● RNA from cells irradiated with 50 krad of X-rays. (B) Determination of DNA nicking and repair in irradiated cells (irradiation at 37°C). O—O DNA from control cells; ●—● DNA from cells irradiated with 20 krad of X-rays; ◐—◐ DNA from cells incubated for 40 min at 37°C after irradiation with 20 krad.

lows that the damage to the template which occurs (nicks, DNA-protein crosslinks, base damage, etc.) is insufficient to inhibit transcription at the given dose of X-rays. One can conclude that *in vivo* inhibition of transcription is due to the sudden stop of RNA-polymerase propagation along the template. It cannot be excluded that initiation is also blocked.

We then determined the size of newly synthesized RNA in irradiated cells. It was found that the size of nascent RNA does not change even at doses which decrease the transcription more than five-fold (Fig. 3A). Hence, the inactivation of the transcriptional units was according to an "all-or-none" principle. This suggests that in undamaged chromatin domains (loops), transcription proceeds normally, but in damaged domains transcription stops completely. (It is interesting to note that after UV-irradiation the size of transcripts diminishes due to the formation of pyrimidine dimers in DNA, which prevent RNA-polymerase propagation /12/). Thus, it follows from our experiments that the nicks themselves (and other DNA and chromatin damage and modification) do not stop transcrip-

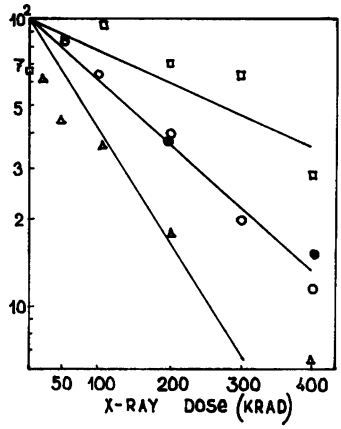

Fig. 4. Inhibition of *in vivo* transcription of different genes in Drosophila melanogaster K_c cells. (○) Total transcription, (●) Ribosomal RNA (18S and 28S) (clone 5X), (▲) Transcription of heat shock gene Hsp 83 (clone 244), (□) Transcription of histone gene cluster of H2A, H2B, H3, H4 and H1 genes (clone p604).

tion, but they may bring about some alteration in the whole unit of transcription.

Independent evidence for the notion that nicks themselves do not prevent transcription was obtained in the course of studies of transcription of individual genes in Drosophila (Fig. 4). No correlation was found between the size of transcript and its sensitivity to inactivation with X-irradiation. (Data on more expanded list of genes will be published elsewere).

The results are consistent with the third above-mentioned possibility that the block of transcription occurs due to the loss of DNA closure resulting in a gross alteration in chromatin structure in a whole domain (loop). This alteration may be connected with refolding of the whole chromatin loop after the loss of torsional strain. It also may be connected with conformational alteration (refolding) of nucleosomes kept unravelled in undamaged chromatin.

Some other explanations of the effect unrelated to violation of topological constraint still persist but seem less likely due to the immediate nature of the reaction of trans-

cription to irradiation. Further experiments reported below support the above explanation.

Determination of DNA-target size for inhibition of transcription

If the loss of topological constraint in a given loop abolishes transcription, then after a dose inducing an average of one nick per loop, one should expect a 63% fall in the level of transcription. This follows from the relationship $m=1-e^{-x}$ (obtained from the Poisson distribution), where x is mean number of breaks per loop, and m is the number of loops obtaining at least one DNA break. Such a decrease in transcription is observed after a dose of 20 krad (irradiation at 37°C). At this dose we measure approximately one nick per 500 kb of single-stranded DNA (Fig. 3B). This would mean that the target of inactivation is 250 kb of double-stranded DNA. This figure is consistent with earlier estimates of a medium loop size in mammalian cells equal to 220 kb /16/. (This estimate was made by comparison of the rates of X-ray induced loss of superhelicity in nucleoids and in phage PM2). We have also performed independent measurements of the rate of X-ray-induced nicking of SV40 DNA within isolated minichromosomes (in the absence of any repair) /17/. The obtained figure of one nick per 100 kb of double-stranded DNA at 10 krad roughly corresponds to our data obtained for CV-1 cells irradiated in the cold (data not shown). That would give an average loop size of 100 kb. (Irradiation in the cold gives a 63% decrease in transcription at 10 krad). However, the rate of restoration of active chromatin structure may lag behind the rate of DNA ligation and this may explain some inconsistency between the figures.

Our calculations of the average DNA loop size are somewhat higher than the 50-100 kb value given in some of the current literature, based either on electron microscopic measurements or on estimates of the percentage of DNA attached to the nuclear skeleton and protected from nucleases. However, it is unclear whether all the attachment sites serve as topological closures *in vivo*. Also, many of these estimates were made using Drosophila cells, which may well have a smaller domain size (see below).

Our estimate of the frequency of X-ray-induced single-strand breaks is significantly lower than some currently reported figures (see /18, 19/). This is because almost all of the reported determinations were made in alkaline solutions which nick the DNA at the alkaline-labile sites induced by radiation (see the comparison of the two methods in /18/). Under neutral pH conditions, however, these sites do not change the DNA topology. Of course, these sites are recognized by the repair machinery and are turned into transient nicks; some equilibrium between the generation and sealing of breaks exists. We observe this equilibrium at a given period after irradiation using the formamide-sucrose gradient determination procedure /18/. (Note that our results imply that alkaline-labile sites themselves do not inhibit transcription. This follows from our run-on assays).

Another point to consider is the possibility of unequal rates of DNA repair in transcriptionally active versus inactive chromatin /20-22/. For example, more rapid incision (or sealing) on active chromatin may yield a higher (or lower) number of DNA nicks soon after irradiation. This would bias our estimation of average loop size because we measure the yield of nicks on total DNA. We employed very short ^{3}H-uridine pulses and immediate cell lysis to minimize these uncertainties. Some possible secondary effects such as ADP-ribosylation thus are also diminished.

Thus, our estimation of target size is consistent with the notion that a single strand breakage event in the DNA of closed DNA loops is responsible for the inactivation of transcription by X-irradiation. Relevant observations by other authors argue that the initiation of replication may also require topological closure of DNA loops /23, 24/.

Inhibition of transcription of individual genes and in diverse organisms differing in the average size of the superhelical DNA loop

Although we have ruled out some possible reasons for the inactivation of transcription unrelated to the violation of topological constraint, we could not exclude all conceivable

explanations. We therefore have approached the problem from the other side. It is clear that if the target of inactivation is a DNA loop, then loops of different length would respond differently to irradiation. So, we have compared the transcription from loops of different sizes in a given cell and also overall transcription in diverse organisms differing in average domain size.

The first approach has advantages because it measures the transcription in the same cellular metabolic backgrown, but there are some difficulties in the assessment of individual loop sizes. Some results in the framework of this approach are shown in Fig. 4. It is seen that the rate of inactivation of transcription in separate genes varies considerably. This argues that these are not some intracellular factors common for every gene that are responsible for the effect. Rather, chromatin structure of individual genes may be involved. Moreover, using the rate of X-ray induced DNA single strand breakage we estimated the target size for the inactivation of ribosomal RNA gene to be around 10 kb which is consistent with the size of ribosomal RNA gene repeating unit (12 kb). The rate of inactivation of histone gene is twice slower which corresponds to 6 kb size of histone gene cluster repeating unit. The target size for heat shock gene Hsp 83 is twice higher than that of ribosomal RNA repeating unit. Thus, topological domain for the heat shock gene may be more than 20 kb long. Different inactivation rates for different genes and correlation of target size with the length of the repeating unit for some genes argue that a single hit per topological domain is responsible for the effect.

In the framework of the second approach, we have compared the levels of inactivation of transcription in cells from various organisms differing in medium DNA loop (replicon) size. The results are presented in Table 1. A good correlation was found between the reported "halo radius" /25/ which is thought to be determined by medium loop size; mean replicon size which is often supposed to reflect the size of separate DNA loops; and our data on the doze required to decrease transcription to 37% (D_{37} - a dose providing on average one hit per active

Table 1. Negative correlation of the X-ray dose required to inhibit transcription with reported DNA halo radii and replicon size in diverse organisms

Organism	Halo radius[22]	Mean replicon size	D_{37} of X-rays (krad)
Drosophila melanogaster (Schneider cells)	6.0 μ	40 kb[29+]	200
Saccharomyces cerevisiae	-	60 kb[30]	100
Monkey CV-1 cells	-	130 kb[31*]	10
Gallus domesticus (erythroblasts)	14.1 μ	200 kb[32*]	5

Irradiation was done on ice. Then ^{3}H-uridine was given for 5 min at a growth temperature of a given organism (to minimize the effect of DNA repair). Reactions were stopped by the addition of 0.5% diethylpyrocarbonate, cells were lysed, RNA was isolated and incorporated radioactivity determined.

+There is also an estimate of the average size of supercoiled loops in Drosophila of about 85 kb /33/.

*The figures for replicon size in higher eukaryotes may actually be even higher /34, 35/.

transcription unit (loop)). The correlation is only a rough one, possibly because of some uncertainties in the determination of mean replicon sizes. Also, DNA repair can modulate the inactivation doses for diverse organisms differently. Finally, the total transcription may represent, mainly, riboso-

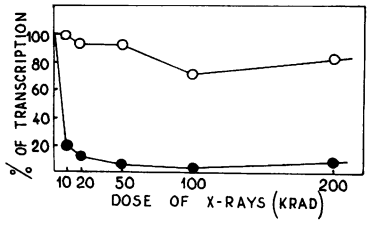

Fig. 5. Dependence of transcription in E. coli (○—○) and in chicken erythroblasts (●—●) on X-ray dose. Irradiation was at 0°C, ^{3}H-uridine was given for 4 min at 37°C after irradiation.

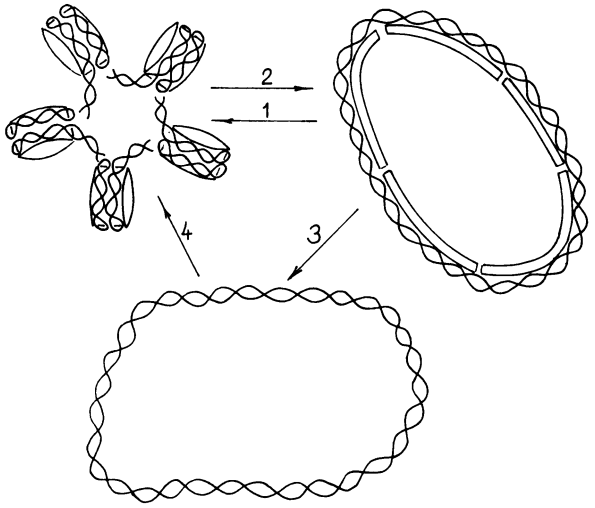

Fig. 6. A model of structural interconversions between active and inactive chromatin. Closed loops are drawn as circles for simplicity.

mal one, while average replicon size may not correspond to ribosomal replicon size. Taking all this into account we conclude that the observed correlation is another piece of evidence that a superhelical DNA loop is a target for inactivation of transcription by ionizing radiation, provided the replicons represent separate topological domains.

When we irradiated E. coli cells, we found no inhibition of transcription (Fig. 5). Even at 200 krad the incorporation of ^{3}H-uridine did not change significantly, notwithstanding the fact that 50 krad is sufficient to relax superhelical DNA in this organism /26/. This result may be explained if up to 70% of bacterial transcription does not respond to changes in DNA torsional strain, as has been reported /27/. The responsive genes do react in different directions /27/. It is interesting to note that in eukaryotic cells heavily contaminated with mycoplasma, we failed to find inhibition of ^{3}H-uridine incorporation by X-irradiation. We believe this is due to the insensitivity of mycoplasmal (prokaryotic) transcription to ionizing radiation.

Thus, it seems likely that all or almost all the transcription in eukaryotic cells depends on topological closure of DNA (and, conceivably, on elastic torsional strain), and that this may be a significant difference from prokaryotes.

The model of transcriptionally-active chromatin

The above results and our earlier data allowed us to elaborate a model of transcriptionally active chromatin and its structural transition in response to DNA relaxation. According to the model, the nucleosomes in transcriptionally active chromatin are extended so as not to interfere with RNA-polymerase passage. The extended nucleosomes are in an unsteady equilibrium which is maintained due to torsional stress in the DNA (Fig. 6). (During the revision of this paper, evidence for torsional-stress-induced nucleosome unfolding has been published /28/). The introduction of a single nick into a closed loop would allow free rotation which would dissipate the elastic strain and facilitate the conformational transition of unfolded nucleosomes into classical globular bodies (Fig. 6, arrow 1). At this stage, temporal dissociation of histones from DNA is not excluded /1/ (Fig. 6, arrows 3, 4). The classical nucleosomes block in vitro transcription at initiation and possibly at elongation steps /13-15/. (A recent paper by Lorch *et al.* /29/ argues that RNA-polymerase may displace histones from short pieces of DNA during *in vitro* transcription. The authors, however, do not rule out "the unfolding of nucleosomes" so that histones could be "released during subsequent gel electrophoresis"). Thus, according to the model, it is not only the elastic strain itself which facilitates transcription, but the chromatin structure maintained by the strain. This point is supported by our *in vitro* transcription experiment in the presence of sarcosyl in which the removal of histones restores the transcriptional competence of irradiated DNA. The possibility of the complete absence of histones in extremely active chromatin (heat shock, or ribosomal RNA genes) should also be considered /30, 31/. In this case, the loss of elastic strain would allow the restoration of

nucleosomes or conformational alterations of non-histone proteins attached to transcriptionally active DNA.

The model requires that torsional-strain is necessary for transcription, but obviously it cannot be sufficient. Indeed, it has been repeatedly shown that genes likely to belong to the same domain can be differentially expressed. Such regulation can be achieved by use of specific initiation factors and may be secondary to the activation of the topological domain during development.

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