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Protein-associated intercalator-induced DNA scission is enhanced by estrogen stimulation in human breast cancer cells

(estrogen receptor/transcriptional chromatin/alkaline elution/anthracyclines/4′-(9-acridinylamino)methanesulfon-*m*-anisidide)

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ABSTRACT **Estrogen-responsive human breast cancer cells (MCF-7) displayed a higher frequency of intercalator-induced protein-associated DNA scission after treatment with 17β-estradiol (E_2) than did cells that had not received estrogen treatment. This effect was dependent on estrogen concentration (maximum enhancement at ≈1 nM E_2) and time (maximum effect seen ≈24 hr after E_2 addition). Human breast cancer cells lacking estrogen receptors did not display the enhanced response. Antiestrogens produced a slight decrease in intercalator-induced DNA scission, whereas insulin produced an enhanced effect. The DNA breaks produced by the intercalators 5-iminodaunorubicin and 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) in these cells were undetectable without enzymatic deproteinization of cell lysates prior to quantification by alkaline elution. Intercalator-induced DNA–protein crosslinking also was enhanced in E_2-treated MCF-7 cells. Studies with *m*-[^{14}C]AMSA revealed no estrogen-associated increases in drug uptake. The data suggest that E_2 treatment, either by specifically and directly increasing active transcription in chromatin or through secondary effects on DNA that accompany alterations in cell growth or cell cycle distribution, alters the susceptibility of DNA to intercalator-induced protein-associated DNA scission. If this enhanced protein-associated scission is selectively localized to transcriptionally active chromatin, the adsorption of the DNA-bound proteins to membrane filters (DNA–protein crosslinking) may allow identification and isolation of estrogen-regulated gene sequences.**

The actions of and cellular responses to a variety of cytotoxic agents involve DNA scission. Ionizing radiation and bleomycin can directly break DNA via putative free-radical mechanisms (1–3). Such breaks may be mechanistically related to the cytotoxic potency of these agents (1, 3–8). Ultraviolet light and a number of chemical agents that result in covalent adducts on DNA bases elicit cellular responses having in common the excision of the DNA containing the exogenous adduct (9). This excision repair is an interruption in the phosphodiester backbone and, therefore, is also a DNA break. These breaks differ from those produced by direct-acting agents as they are part of a response to DNA damage rather than the damage itself. The DNA breaks produced by most exogenous physical or chemical agents are analogous to one of these two types of DNA scission.

We have described a third type of cellular DNA scission. It is produced in cells or isolated nuclei treated with a variety of DNA intercalating agents (10–15). Several characteristics of these breaks suggest that they are formed by endogenous endonucleases responding to intercalator-induced topological alterations in DNA helical structure. Further, substances that alter DNA three-dimensional structure also alter the quantitative, but not the qualitative, intercalator response of murine leukemia cell DNA *in vivo* (7).

The expression of specific gene products must begin with a change in DNA structure. The DNase hypersensitivity of chromatin-containing specific gene sequences substantiates the concept that active or potentially active chromatin differs in structure from the bulk of genomic DNA (16). Alterations in DNA methylation at gene sites may correlate with altered transcription rates (17). This alteration in two-dimensional DNA structure may translate into three-dimensional alterations yielding DNA with physical properties different from DNA that is not actively transcribed (18, 19). Because the magnitude of the DNA scission produced by intercalators can vary with alterations in DNA three-dimensional structure (7), we wondered whether a comparison of this response in cells possessing various amounts of actively transcribing chromatin might serve to identify those cell populations with transcriptionally active chromatin. We report here that a line of estrogen-responsive human breast cancer cells has more intercalator-induced DNA breakage after estrogen treatment than without hormone treatment. Hormonally unresponsive cells are not similarly affected.

MATERIALS AND METHODS

Cell Culture, Radioactive Labeling, and Hormone and Drug Treatments. MCF-7, ZR-75-1, HST-578, and MDA-MB-231 human breast cancer cells were grown in improved minimal essential medium supplemented with 5% fetal bovine serum (improved ME medium A) in 5% CO_2 as described (20, 21). Twenty-four hours prior to labeling, cells were switched to medium supplemented with 5% dextran-coated charcoal-treated calf serum (improved ME medium B), a procedure that substantially reduces endogenous steroid concentrations (20). The DNA of these cells was labeled with [^{14}C]thymidine (53.4 mCi mmol^{-1}, New England Nuclear; 1 Ci = 37 GBq) at a final concentration of 0.05 μCi ml^{-1} for about 20 hr, followed by an incubation in label-free improved ME medium B for at least 3 hr prior to the beginning of any hormone or drug additions. Mouse leukemia L1210 cells grown in RPMI 1630 medium supplemented with 15% fetal calf serum and labeled for 20 hr with [*methyl*-^{3}H]thymidine (20 Ci mmol^{-1}, New England Nuclear; final concentration, 0.1 μCi ml^{-1} with 1 μM unlabeled thymidine added) served as internal standard cells for alkaline elution assays as described (22, 23).

Stock solutions of 17β-estradiol and tamoxifen were in ethanol at 1,000-fold the final desired concentration. LY117018 ethanol solution was 500-fold the final desired concentration. Insulin

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Abbreviations: E_2, 17β-estradiol; SSB, DNA single-strand break; *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide.

(U-100, regular Iletin I, Eli Lilly) was diluted 1:1,000 for cell treatment. 5-Iminodaunorubicin (NSC 254681) was a gift from R. I. Glazer (Applied Pharmacology Section, Laboratory of Medicinal Chemistry and Biology, National Cancer Institute), who obtained the compound from E. Acton (Stanford Research Institute), and 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) base (NSC 249992) was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Both compounds were kept as frozen stock solutions: 5-iminodaunorubicin at 1 mM in sterile, glass-distilled water and m-AMSA at 10 mM in sterile dimethyl sulfoxide. All drug treatments were for 30 min at 37°C.

DNA Alkaline Elution. The methodology of alkaline elution has been detailed in previous publications (14, 22, 23). Briefly, cells for analysis were rinsed free of drug with iced Hanks balanced salt solution and removed from tissue culture dishes by mechanical scraping with a rubber policeman.

[^{14}C]Thymidine-labeled breast cancer cells ($\approx$1–5 $\times$ 10^5) were mixed with 5 $\times$ 10^5 [^{3}H]thymidine-labeled internal standard L1210 cells and deposited on 2-μm polycarbonate filters (Nucleopore) in a Swinnex-25 filter holder (Millipore). Lysis was effected with 2% $NaDodSO_4$ (BDH) in 0.1 M glycine/0.025 M Na_2EDTA, pH 10 (5 ml). DNA was slowly eluted (flow rate, 0.03–0.04 ml min^{-1}) from these filters with 2 ml of proteinase K (Merck; at 0.5 mg ml^{-1} in the $NaDodSO_4$ lysis solution) followed by 35 ml of 0.1 M tetrapropylammonium hydroxide (RSA, Ardsley, NY)/0.02 M EDTA/0.1% $NaDodSO_4$, pH 12.1. Samples were collected at 3-hr intervals for 15 hr, and the radioactivity in each sample, in the $NaDodSO_4$ cell lysate, and on the filter was quantified by liquid scintillation spectrometry.

The rate of DNA elution is linearly related to the frequency of DNA single-strand breakage (SSB) as determined by an x-ray standard curve (see refs. 14, 22, and 23). [^{3}H]Thymidine-labeled internal standard cells receiving 300 R of x-radiation at 4°C prior to their deposition on the filter provide a uniform DNA elution rate against which the elution of human cell DNA can be plotted. This minimizes the effects of variation in elution rate from experiment to experiment. Intercalator-induced DNA SSB frequency was quantified as described (10–15) by comparing the elution rate of DNA from drug-treated cells with that from cells receiving a known quantity of x-radiation. This SSB frequency was calculated by the formula

$$P_{BD} = \frac{\log (r_1/r_o)}{\log (R_o/r_o)} P_{BR}$$

where P_{BR} is the DNA break frequency produced by x-radiation (200 rad equivalents) and r_1, r_o, and R_o are the retentions of [^{14}C]DNA from drug-treated, untreated, and 200-R x-irradiated cells. This retention is the fraction of [^{14}C]DNA remaining on the filter when 35% of the [^{3}H]DNA remains on the filter. P_{BD} (SSB frequency) is expressed in rad equivalents. The ratio of the SSB frequencies in hormone-treated over untreated cells within a single experiment is the "DNA scission ratio." The frequency of DNA–protein crosslinking and DNA breaks detectable without proteolytic enzymes were quantified by alkaline elution as described (14, 22, 23).

***m*-[^{14}C]AMSA Uptake.** *m*-[^{14}C]AMSA (19.6 mCi $mmol^{-1}$) was synthesized by SRI International (Menlo Park, CA) and was obtained through the Chemical Resources Section, National Cancer Institute. MCF-7 cells were mechanically dislodged from their plates, concentrated into 2 ml of fresh improved ME medium B at 10^6 cells ml^{-1} in 15-ml plastic centrifuge tubes. These cells were incubated with 0.5 μM *m*-[^{14}C]AMSA and 2 μl of tritiated H_2O (1 mCi ml^{-1}; New England Nuclear) at 37°C for 30 min. The uptake of *m*-AMSA per cell volume (as quantified by the distribution of ^3H_2O) was measured by pelleting the cells through Versilube F-50 silicone oil in a microcentrifuge tube at 12,000 $\times$ *g* for 1 min, cutting the bottom off of the tube, and dispersing and solubilizing the cell button in a liquid scintillation vial. Corrections for extracellular material trapped in the pellet were made by the estimation of this quantity per cell volume by using [^{14}C]inulin (New England Nuclear) in separate experiments (See ref. 14). This technique is an adaptation of that of Vistica (24).

Table 1. The enhanced intercalator-induced DNA scission produced in MCF-7 human breast cancer cells after 19–24 hr of treatment with 1 nM E_2*

Intercalator	Dose, μM $\times$ 30 min	DNA scission ratio†	*P* value‡
5-Iminodaunorubicin	5	1.57 ± 0.08 (6)	0.028
	2.5	1.59 ± 0.13 (10)	0.005
m-AMSA	0.5	1.32 ± 0.26 (5)	0.043
	0.25	1.40 ± 0.44 (7)	0.018

* MCF-7 cells were incubated with or without E_2 between 19 and 24 hr at 37°C prior to the addition of either intercalator for 30 min.
† The ratio of the SSB frequency in estrogen-treated vs. untreated cells after the 30-min intercalator treatment ± 1 SD (number of experiments shown in parentheses).
‡ Two-sided *P* value by signed-rank test.

RESULTS

The DNA scission produced in MCF-7 cells by either 5-iminodaunorubicin or *m*-AMSA was significantly greater in E_2-stimulated cells than in unstimulated cells (Table 1). The SSB frequency in E_2-treated cells was about 60% higher after addition of 5-iminodaunorubicin and about 35% higher after addition of *m*-AMSA. This enhancement of SSB frequency was

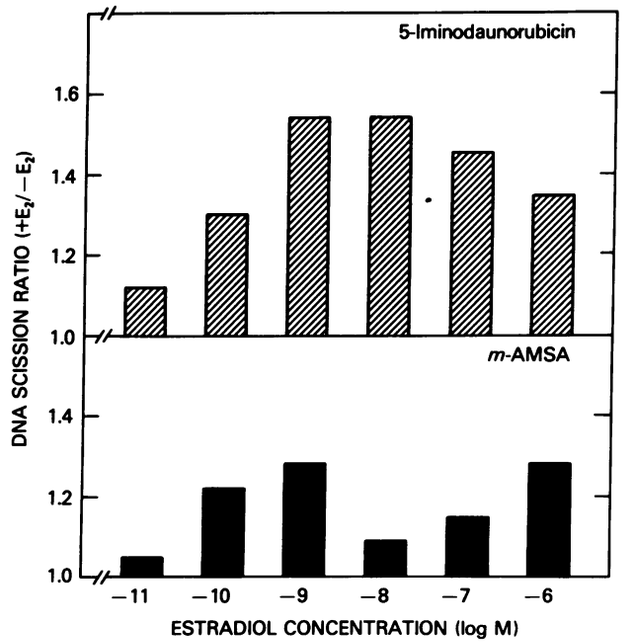

FIG. 1. The enhancement of intercalator-induced DNA scission in MCF-7 cells after treatment with various concentrations of E_2. MCF-7 cells were treated with E_2 for 21–22 hr, followed by a 30-min treatment with either 2.5 μM 5-iminodaunorubicin (*Upper*) or 0.25 μM *m*-AMSA (*Lower*), after which DNA SSB frequency was quantified by alkaline elution. The ordinate is the ratio of the SSB frequency produced in estrogen-treated vs. untreated cells. The data for 5-iminodaunorubicin is the mean of two independent experiments.

Table 2. The effects of other hormones on intercalator-induced DNA scission*

Experiment	Hormone (Dose)	Intercalator	Dose, μM × 30 min	DNA scission ratio†	Enhanced DNA scission by E_2‡
1	Tamoxifen (2 μM × 21 hr)	5-Iminodaunorubicin	5	0.88	1.44
			2.5	0.81	1.41
		m-AMSA	0.25	0.88	1.41
2	Tamoxifen (1 μM × 22 hr)	5-Iminodaunorubicin	5	0.90	1.67
	LY117018 (20 nM × 22 hr)	5-Iminodaunorubicin	5	0.91	1.67
	Insulin (0.1 unit ml^{-1})	5-Iminodaunorubicin	5	1.36	1.67

* Each hormone treatment was for 21–22 hr prior to the addition of the intercalator for 30 min.
† The ratio of SSB frequency in hormone-treated vs. untreated cells after the 30-min intercalator treatment.
‡ E_2 treatment was 1 nM for 21–22 hr in experiments performed concurrently with those using other hormones.

dependent on E_2 concentration (Fig. 1), with little effect at 10 pM E_2 and a maximum effect at 1 nM E_2. The SSB frequency produced by 5-iminodaunorubicin was lower in cells treated with E_2 at concentrations in excess of 10 nM than at the lower E_2 concentrations but still exceeded that seen in cells not receiving E_2. The DNA from cells treated with E_2 alone was eluted like that from control cells.

The antiestrogens tamoxifen or LY117018 significantly diminished intercalator-induced DNA scission in MCF-7 cells ($P = 0.04$) (Table 2), whereas insulin stimulated DNA scission. Human breast cancer cells lacking estrogen receptor (MDA-MB-231) did not exhibit this estrogen-enhanced DNA scission after additions of either 5-iminodaunorubicin or *m*-AMSA (Table 3). Qualitatively similar results to the MCF-7 and MDA-MB-231 data were obtained with ZR-75-1 and HST-578, two lines possessing and lacking estrogen binding protein, respectively. MCF-7 and MDA-MB-231 did differ in the amount of DNA scission produced by a given drug concentration (Table 4). This was not due to differences in sensitivity to the breakage produced by x-radiation against which drug-induced scission is calibrated (see refs. 22 and 23). However, over a range of drug concentrations producing comparable *m*-AMSA-induced DNA scission in cells

Table 3. The effect of estrogen treatment (1 nM for 20–22 hr) on the intercalator-induced DNA scission produced in MDA-MB-231*

Intercalator	Dose, μM × 30 min	DNA scission ratio†
5-Iminodaunorubicin	5	0.86
	2.5	1.08
	2	0.67
	1	0.98
m-AMSA	0.5	0.91
	0.25	0.89
	0.20	0.77
	0.10	0.76, 1.09
	0.05	1.10, 0.88

* A human breast cancer cell line lacking estrogen receptor.
† The ratio of the SSB frequency in estrogen-treated vs. untreated cells after the 30-min intercalator treatment.

Table 4. The production of DNA scission by x-radiation or *m*-AMSA treatment in MCF-7 or MDA-MB-231 human breast cancer cells

Cell line	X-radiation at 200 R*	*m*-AMSA for 30 min at 37°C†, rad equivalents: 0.5 μM	0.25 μM	0.2 μM	0.1 μM
MCF-7	0.29 ± 0.08 (11)	224.9 ± 80.1 (5)	188.6 ± 78.1 (6)	—	—
MDA-MB-231	0.33 ± 0.01 (4)	395.9	326.7	244.1	135.6 192.4

* Results are the fraction of irradiated breast cancer cell DNA ([^{14}C]dT-labeled) remaining on the filter when 35% of the 300-R-irradiated internal standard L1210 cell DNA ([^{3}H]dT-labeled) remains on the filter and are expressed as the mean ± SD (number of determinations shown in parentheses).
† Results in rad equivalents are expressed as the mean ± SD if more than two experiments (number shown in parentheses) were performed.

not receiving E_2 treatment, the cells possessing E_2 receptors exhibited an enhanced susceptibility to *m*-AMSA-induced scission after E_2 treatment, whereas E_2 receptor-negative cells did not (Tables 1 and 3).

One possible explanation for these results would be an enhanced uptake of intercalator into estrogen-treated cells either through alterations in cell membranes or alterations in intracellular chemistry favoring enhanced drug influx. However, direct measurements of MCF-7 uptake of *m*-[^{14}C]AMSA did not indicate alterations in *m*-[^{14}C]AMSA uptake produced by E_2 treatment (Table 5). However, insulin did enhance the uptake of *m*-[^{14}C]AMSA (Table 6) ($P = 0.04$) and may explain the enhanced 5-iminodaunorubicin-induced DNA scission seen after treatment with this hormone (Table 2), if the uptake of 5-iminodaunorubicin resembles that of *m*-AMSA.

In Fig. 2 the development of the enhanced 5-iminodaunorubicin-induced SSB frequency with time of E_2 treatment is shown. In some experiments (data not shown), small increases were noted as early as 1 hr after estrogen addition, but prolonging the E_2 treatment clearly magnified this effect. The time course of the development of E_2-enhanced intercalator-induced scission was consistent with the time course for E_2 stimulation of nucleic acid precursor uptake and the synthesis of specific estrogen-dependent translatable mRNA (20, 25, 26).

Finally, it is important to stress that the drug-induced SSBs were protein-concealed—that is, nondetectable in assays without proteolytic enzyme digestion both in E_2-treated and untreated cells. Further, in assays quantifying DNA–protein crosslinking (see refs. 14, 22, and 23), the magnitude of the enhancement of intercalator-induced DNA–protein crosslink frequency seen in E_2-treated cells mirrored the magnitude of protein-associated strand break enhancement after addition of 5-iminodaunorubicin or *m*-AMSA.

Table 5. The effect of estrogen (1 nM) treatment on the uptake of *m*-[^{14}C]AMSA in MCF-7 human breast cancer cells*

Experimental condition	Calculated intracellular *m*-AMSA concentration, μM ± 1 SD
m-AMSA alone	3.77 ± 1.13
Pretreatment with E_2 × 21 hr	3.72 ± 0.16
Pretreatment with E_2 × 30 min	4.26 ± 0.72

* MCF-7 cells were incubated with m-AMSA at 0.5 μM for 30 min after their removal from plates, and radioactive drug uptake was quantified by modifications (14) of the method of Vistica (24).

Table 6. The effect of insulin (0.1 unit ml^{-1}) treatment on the uptake of *m*-[^{14}C]AMSA in MCF-7 human breast cancer cells*

Experimental condition	Calculated intracellular *m*-AMSA concentration, μM ± 1 SD
m-AMSA alone	3.53 ± 0.56
Pretreatment with insulin × 22 hr	6.06 ± 0.67
Pretreatment with insulin × 30 min	3.19 ± 0.52

* MCF-7 cells were incubated with *m*-AMSA at 0.5 μM for 30 min after their removal from plates, and radioactive drug uptake was quantified by modification (14) of the method of Vistica (24).

DISCUSSION

Protein-associated DNA scission is a newly described cellular response to DNA intercalation. Several aspects of this response support its being enzymatically mediated but different from those enzymatic responses that are elicited in cells treated with ultraviolet light, alkylators, or other agents that produce covalent DNA adducts followed by excision repair (10–15).

Alterations in the compaction of chromatin (nucleoid sedimentation) correspond to alterations of the magnitude of this response in murine cells (7). Therefore, this response appears to depend on the three-dimensional structure of the DNA target and may be a response to helical distortion produced by intercalation. Topoisomerases are enzymes capable of altering three-dimensional DNA structure and relieving torsion by covalently binding to DNA (DNA–protein crosslink) and producing a break in the DNA backbone, followed by topological rearrangement of the DNA and resealing of the break (27). Intercalator-induced, protein-associated DNA scission may be a topoisomerase-mediated cellular response to helical torsion. The magnitude of this response to torsion may be governed by the three-dimensional configuration of the DNA at the time of intercalator addition. Our previous work demonstrating nonspecific chemical perturbations of nuclear compaction correlating with the magnitude of this response supports this idea (7).

In the present work, steroid hormones were used, in part because of presumed specific sites of interaction with chromatin resulting in gene transcription. Actively transcribing chromatin has been found to have altered sensitivity to nuclease digestion and, thus, probably possesses a structure in some fashion distinct from the bulk of the cellular chromatin (16). E_2-responsive cells treated with E_2 and, thus, presumably with an increased amount of actively transcribing chromatin, like cells with less compacted chromatin (7), display more intercalator-induced DNA scission than do untreated cells. As drug uptake appeared comparable (Table 5), several possible mechanisms could explain this finding.

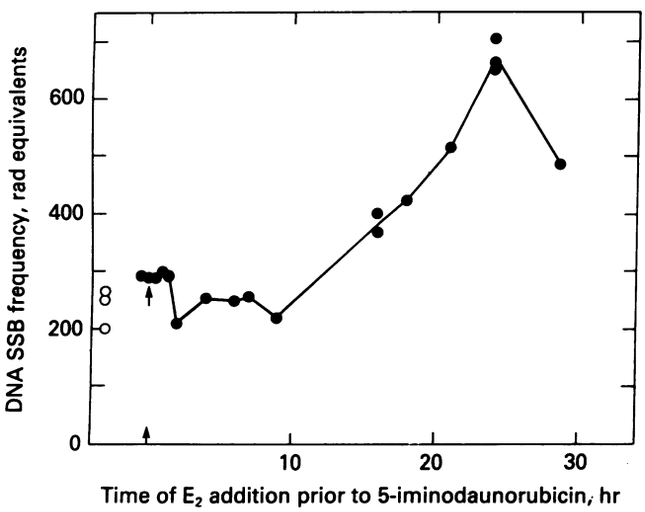

FIG. 2. The time course of estrogen enhancement of intercalator-induced DNA scission in MCF-7 cells. Cells were treated with E_2 (1 nM) for various times (●) prior to the addition of 2.5 μM 5-iminodaunorubicin for 30 min. DNA SSB frequency was quantified by alkaline elution and expressed in rad equivalents. Arrow, time of 5-iminodaunorubicin addition; ○, SSB frequencies produced in replicate plates treated with 5-iminodaunorubicin with no added estradiol.

Cells or sites within cells after hormonal treatment could possess more of the enzyme that putatively produces the DNA scission. Recent work has, indeed, identified topoisomerase I activity as a component of nucleosomes from actively transcribing genes (28). Thus, enhanced intercalator-induced scission might be expected in cells with enhanced gene transcription.

Alternatively, as E_2 not only causes specific gene transcription but also increases cell growth (26, 29, 30), the enzyme may increase in cells with reduced doubling times. As cells with rapid doubling times might require more of an enzyme putatively required for DNA unwinding prior to synthesis, this, too, would not be surprising. Additionally, as with transcriptionally active chromatin, the chromatin of rapidly dividing or cycling cells could differ in structure from that of slower growing cells and possess an altered susceptibility to intercalator-induced scission. However, recent work in our laboratory has shown that the cell cycle-perturbing antimetabolites arabinosylcytosine or hydroxyurea, which prolong cell doubling times, also can potentiate intercalator-induced DNA scission (31), tending to mitigate against a simple relationship between enhanced cell growth rate by E_2 and hormonally stimulated susceptibility to intercalator-induced DNA scission. However, because these antimetabolites tend to accumulate cells in the S phase of the cell cycle (31), DNA structural or biochemical correlates may exist between these cells and rapidly growing E_2-stimulated ones, which result in both cases in enhanced intercalator-induced DNA scission. Thus, we cannot eliminate effects of estrogen treatment on cell growth rate or cell cycle distribution as the source of enhanced intercalator-induced DNA scission.

Insulin, which increases MCF-7 growth to a greater extent than does E_2 (29) and which probably indirectly stimulates specific gene transcription of its own, will not aid in distinguishing between these potential alternative models, as it also can enhance *m*-AMSA (Table 5) and ellipticine (32) cellular uptake. However, this hormone did serve as a valuable control, demonstrating that although hormonal stimulation of cells can alter drug uptake in the case of insulin, this is not necessarily so for all hormones.

Actively transcribing chromatin may possess additional intercalation sites, putatively at or near the genes themselves potentially analogous to the nuclease-sensitive sites described by others (16). Thus, although intracellular drug concentration may be unchanged by E_2, more drug may be intercalated into the DNA of cells with more actively transcribing chromatin and, thus, increase the helical distortion produced by intercalation and the concomitant cellular response to this distortion.

Alternatively, the actual number of intercalation sites may be identical in more or less actively transcribing cells. However, the structure of actively transcribing chromatin which leads to nuclease hypersensitivity also may allow the propagation of the intercalator-induced helical distortion over a greater length of DNA. If stable topoisomerase sites exist, as has been speculated (33–35), then for any given intercalator-induced distortion, more sites might be recruited in cells with more transcribing genes than in those with less transcriptionally active chromatin because of this wider propagation giving rise to more

breaks. In either of these last two models, if gene expression is accompanied by specific undermethylation of critically located cytosines (17), a possible shift from Z- to B-DNA configuration, which can accompany hypomethylation and gene expression, could occur. If Z-DNA serves as a safety valve to dampen the effect of intercalator-induced helical distortion, the conversion of Z-DNA to B-DNA (18, 19), which could accompany gene expression, might favor either the enhancement of intercalation at gene sites (B-DNA would probably be a better intercalative target) or a wider propagation of helical torsion or both.

If some or all of the increased number of intercalator-induced breaks seen after hormonal treatment are at specific gene sites, then covalent bonds between a protein (the enzyme) and one end of the broken strand exist at these specific sites (DNA–protein crosslinking). It may be possible after intercalator treatment to selectively adsorb onto filters protein-bound DNA containing specific, actively transcribing genes.

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