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Hormonal Induction of Transfected Genes Depends on DNA Topology

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Plasmids containing the hormone regulatory element of mouse mammary tumor virus linked to the thymidine kinase promoter of herpes simplex virus and the reporter gene chloramphenicol acetyltransferase of *Escherichia coli* respond to glucocorticoids and progestins when transfected into appropriate cells. In the human mammary tumor cell line T47D, the response to progestins, but not to glucocorticoids, is highly dependent on the topology of the transfected DNA. Although negatively supercoiled plasmids respond optimally to the synthetic progestin R5020, their linearized counterparts exhibit markedly reduced progestin inducibility. This is not due to changes in the efficiency of DNA transfection, since the amount of DNA incorporated into the cell nucleus is not significantly dependent on the initial topology of the plasmids. In contrast, cotransfection experiments with glucocorticoid receptor cDNA in the same cell line show no significant influence of DNA topology on induction by dexamethasone. A similar result was obtained with fibroblasts that contain endogenous glucocorticoid receptors. When the distance between receptor-binding sites or between the binding sites and the promoter was increased, the dependence of progestin induction on DNA topology was more pronounced. In contrast to the original plasmid, these constructs also revealed a similar topological dependence for induction by glucocorticoids. The differential influence of DNA topology is not due to differences in the affinity of the two hormone receptors for DNA of various topologies, but probably reflects an influence of DNA topology on the interaction between different DNA-bound receptor molecules and between receptors and other transcription factors.

The long terminal repeat region of the mouse mammary tumor virus (MMTV) contains nucleotide sequences that are specifically recognized by glucocorticoid and progesterone receptors and mediate the inducibility of adjacent promoters by these hormones (for a review, see reference 4). These sequences have been called hormone regulatory (or responsive) elements (HREs). The mechanism by which binding of hormone receptors to the HRE influences promoter utilization is not known. As for other eucaryotic regulatory proteins, two mechanisms have been considered: a direct interaction of DNA-bound regulatory proteins with other transcriptional control factors (22), and a possible influence of specific protein-DNA interactions on the structure of chromatin (1, 2, 8). To explain effects at a distance, characteristic of eucaryotic enhancer elements, additional assumptions are needed in both models. For the protein-protein interaction, model looping out of the intervening DNA must be postulated and has been observed in some systems (22). If a conformational change of chromatin is induced by binding of a regulatory protein to the enhancer region, one must envisage mechanisms that would allow this effect to propagate along the nucleosomally organized chromatin to reach the promoter region. In both cases the topology of the DNA domain containing the regulatory elements and the promoter could influence the communication between proteins bound to the two regions. For instance, negative supercoiling has been shown to decrease the energy required for DNA loop formation (16).

In gene transfer experiments, as well as following microinjection into *Xenopus* oocytes, the expression of plasmids containing enhancer elements has been shown to be dependent upon the initial DNA topology, in that supercoiled plasmids are transcribed more efficiently than their linear counterparts (13, 28). Constructs that contain the simian virus 40 enhancer physically linked to, but topologically isolated from, the β-globin promoter show fivefold-lower, although still significant, activity in gene transfer experiments (21). The enhancer effect is also markedly reduced when psoralen-modified DNA is inserted between the enhancer and the promoter (9). These findings suggest that the mechanism by which enhancer elements activate adjacent promoters depends on an organization of DNA in chromatin that favors supercoiled DNA.

It has been shown that in cells carrying minichromosomes containing the long terminal repeat region of MMTV, the nucleosomes around the relevant region of the promoter are phased in such a way that a nucleosome, or a nucleosome-like structure, covers the HRE region (23). Upon induction by glucocorticoids, the accessibility of the promoter region to nucleases and transcription factors is increased (8). In cells containing chromosomally integrated MMTV constructions, addition of dexamethasone leads to the appearance of a DNase I-hypersensitive area overlapping with the region known to be recognized by the glucocorticoid receptor in vitro (29). Since the organization of circular DNA into chromatin could be markedly influenced by DNA topology (27), we have analyzed the influence of the initial topological state of plasmids containing the HRE of MMTV linked to the thymidine kinase (TK) promoter of herpes simplex virus on the response to glucocorticoids and progestins after transfection into appropriate cells. We found that optimal induction by progestins in a clone of the human mammary tumor cell line T47D is seen only with negatively supercoiled plasmids and not with linear DNA molecules, whereas glucocorticoids induce linear and supercoiled plasmids equally well. These results are not due to differences in the

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transfection efficiency obtained with plasmids of different topology, nor do they simply reflect a differential affinity of each hormone receptor for the various topological forms of the DNA. On the basis of results obtained with different insertion mutants, we propose that DNA topology determines the efficiency with which proteins bound to the HRE and to the promoter of MMTV interact to activate transcription.

MATERIALS AND METHODS

Cell lines and transfection procedures. T47D cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and 0.6 μg of bovine insulin per ml. XC cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Cell transfections were carried out by the DEAE-dextran method as previously described (6). One million T47D cells were seeded onto 6-cm culture plates, and 24 h later the cells were washed twice with Tris-buffered saline (TBS) and incubated at room temperature for 30 min with a mixture containing 1 μg of supercoiled or linearized plasmid, 1 μg of pRSV-lacZ, and, when indicated, 1 μg of pRSV-GRcDNA (19). The total amount of transfected DNA was made up to 5 μg by the addition of supercoiled pBR322. After incubation the cells were washed once with TBS and treated with 20% dimethyl sulfoxide in culture medium for 2 min at room temperature. The cells were washed with TBS and incubated under 5% CO_2 for 5 h at 37% in culture medium containing 0.1 mM chloroquine diphosphate. The cells were then washed with TBS and further incubated with culture medium for 24 h before hormones were added (100 nM dexamethasone or 30 nM R5020). After additional incubation for 40 h in culture medium at 37°C and 5% CO_2, the cells were harvested and chloramphenicol acetyltransferase (CAT) assays were performed with 20 μg of protein as described previously (6, 11). The proportion of radioactive chloramphenicol that was acetylated was measured by using a radioactivity scanner (Bioscan; Packard Instrument Co., Inc.), from which the enzyme activity was calculated. Small variations in transfection efficiency occurring within an experiment were monitored with β-galactosidase as an internal standard. The normalized values are expressed as picomoles of chloramphenicol acetylated per minute per milligram of protein.

Plasmids. The plasmid pMMTV-TK-CAT corresponds to the previously described pTK-CAT-5A (5). pLS-147, pBMI-5, and pBMI-10, containing 5- and 10-base-pair (bp) insertions between the receptor binding sites of the HRE, have been previously described (7). The insertions between the HRE and the TK promoter (pBHI mutants) were introduced in the unique *Bam*HI site present in pMMTV-TK-CAT. pBHI-4, a 4-bp insertion, was obtained by linearizing with *Bam*HI, filling the overhanging ends with the Klenow fragment of DNA polymerase, and religating with T4 DNA ligase. pBHI-37, a 37-bp insertion, was obtained by introducing in the *Bam*HI site a 37-bp synthetic polylinker with *Bam*HI overhanging ends (5′-GATCCCGGGAAGCTTG ATATCGATACCCGGGATATCC-3′), containing two *Sma*I sites, two *Eco*RV sites, and one *Hin*dIII site. Subsequent digestion of this plasmid with *Eco*RV (pBHI-22) or *Sma*I (pBHI-15) or both (pBHI-10) and religation yielded the different insertion mutants. *Hin*dIII digestion and further religation of pBHI-37, which contains two *Hin*dIII sites, one belonging to the parental pMMTV-TK-CAT at position −456 and the second located in the polylinker at 34 bp downstream from the last binding site, gave in 50% of the clones a derivative containing this 397-bp *Hin*dIII fragment in the inverted orientation respective to pMMTV-TK-CAT, the so-called pINV.

DNA preparation. Supercoiled DNA was obtained from CsCl gradients in the presence of ethidium bromide. Linear plasmids were obtained by digestion with *Aat*II (at position −920). Linear DNA was separated from undigested species by preparative agarose gel electrophoresis (1% agarose) and electroelution. In some cases, the 3′ *Aat*II overhanging ends were removed with the Klenow fragment of DNA polymerase I in the presence of 50 μM ddGTP. Extensive treatment of the resulting linear DNA with T4 DNA ligase gave no detectable circular forms. Nicked DNA was obtained by digestion of supercoiled plasmids with DNase I in the presence of 200 ng of ethidium bromide per μl (12). Relaxed DNA was obtained by religation of digested plasmids with *Hin*dIII T4 DNA ligase. Radioactive supercoiled pMMTV-TK-CAT was obtained by religation of the 5′-end-labeled, *Hin*dIII-linearized plasmid with T4 DNA ligase in the presence of 2 ng of ethidium bromide per μl. When necessary, the desired species were isolated from a preparative 1% agarose gel and electroeluted.

DNA-binding assays. The relative affinities of hormone receptors for different topological forms were measured with supercoiled and linear labeled pMMTV-TK-CAT. A mixture containing equal amounts (4 ng) of both forms was incubated for 45 min at 0°C with 400 pg of progesterone receptor or 6 ng of glucocorticoid receptor (yielding about 20% binding in both cases) and filtered through a nitrocellulose filter. The bound DNA molecules were eluted from the filter by incubation for 30 min at 37°C with moderate shaking in 50 μl of TE buffer (10 mM Tris hydrochloride [pH 7.4], 1 mM EDTA) containing 0.1% sodium dodecyl sulfate and 5 μg of tRNA per ml. The eluted DNA was analyzed by electrophoresis in a 1% agarose phosphate-buffered gel, which was dried and exposed overnight on Kodak X-Omat film.

Analysis of transfected DNA. For quantification of transfected DNA, T47D cells were transfected with 2 μg of either supercoiled or linear pMMTV-TK-CAT, 2 μg of supercoiled pUC8, and 6 μg of carrier calf thymus DNA in 10-cm-diameter plates. The cells were harvested 24 h after transfection, the culture medium was removed by two washes with TBS, and the cells were resuspended in 1 ml of TBS containing 1 mM $MgCl_2$. DNA adsorbed to the cells was digested with 50 μg of DNase I for 30 min at 37°C. The digestion was terminated by the addition of 20 μl of 0.5 M EDTA and chilling on ice. The cells were washed twice with TBS containing 1 mM EDTA and finally suspended in 750 μl of 0.25 M sucrose in TES buffer (15 mM Tris hydrochloride [pH 7.4], 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 2.5 mM EDTA) containing 0.25% Nonidet P-40. After repeated pipetting to break the cell membranes, the nuclei were sedimented at 5,000 × *g* for 10 min at 4°C, and the pellet was resuspended in the same buffer and recentrifuged. This second pellet was washed twice with 0.25 M sucrose in TES buffer, and the nuclear pellet was finally suspended in TE buffer. Total DNA was extracted by digestion with 50 μg of proteinase K per ml in 0.1% sodium dodecyl sulfate–1 M NaCl for 4 h at 37°C, followed by phenol-chloroform-isoamyl alcohol extraction and further precipitation with 2.5 volumes of ethanol in 0.2 M NaCl. The purified DNA was digested with *Bam*HI, and the result of the digestion was analyzed by agarose gel electrophoresis (1% agarose). The DNA was blotted onto a GeneScreen (Du Pont, NEN Research Products, Boston, Mass.) and hybridized with pMMTV-TK-CAT labeled by nick translation (10), with a specific activity

higher than 3×10^4 dpm/ng. The membranes were exposed for 18 to 48 h on Kodak X-Omat film with two intensifying screens.

For the analysis of transfected DNA, T47D cells were transfected with 2 μg of either supercoiled or linear pM MTV-TK-CAT DNA, harvested, and digested with DNase I as described above. Nuclease-treated cells were suspended at 10^7 cells per ml in 50 mM Tris hydrochloride–10 mM EDTA (pH 7.5) and mixed gently with 1 volume of 1.2% sodium dodecyl sulfate and 0.5 volume of 5 M NaCl, and Hirt supernatants were prepared (14). Following phenol-chloroform-isoamyl alcohol (25:24:1) extraction and ethanol precipitation in the presence of carrier tRNA, the recovered material was separated in 1% agarose gels, transferred to nylon membranes, and hybridized with an *Nco*I-*Bgl*II fragment from the CAT gene labeled by random priming (Amersham Corp.) to a specific activity of approximately 10^5 dpm/ng.

Analysis of the transcription start point. Transcription start sites for the TK-CAT fusion mRNA were determined by using an RNase protection procedure and uniformly labeled antisense RNA probes as previously described (6, 18, 30).

RESULTS

Influence of DNA topology on the response to progestins. We have previously shown that plasmids containing the HRE of MMTV linked to the TK promoter and the CAT gene of *Escherichia coli* respond to progesterone induction after transfection into the mammary cell line T47D (5). In these experiments, negatively supercoiled plasmids were used for the transfection, since they are known to yield optimal results (12). To test whether the hormonal response is influenced by the topology of the transfected plasmid, we compared results obtained with supercoiled and linear plasmids. For this purpose, plasmids were digested with single-cut restriction enzymes, and the linearized as well as the undigested supercoiled forms were isolated by agarose gel electrophoresis prior to transfection. The CAT activity measured in the absence of hormone was very low and was not influenced by the topology of the plasmid (Fig. 1B insert), whereas the response to the synthetic progestin R5020 was 7 to 10 times better with negatively supercoiled plasmids. The results of a typical experiment are shown in Fig. 1. Although the total level of CAT activity varied among experiments as a result of changes in transfection efficiency, the observed differences between the topological forms of the plasmid were highly reproducible. In an average of five separate experiments, the induction observed with linearized plasmid was 13.2% ± 3.5% of that found with the corresponding supercoiled construct. The TK promoter was correctly transcribed from both linear and supercoiled plasmids, as demonstrated by RNase protection analysis of the transcription start point (Fig. 2). Thus, we conclude that the use of negatively supercoiled plasmids is required for optimal progesterone induction of constructions containing the HRE of MMTV. In that respect, the progesterone-induced MMTV enhancer behaves similarly to the Rous sarcoma virus enhancer (28) (Table 1).

Influence of DNA topology on the response to glucocorticoids. Next, we wanted to know whether the response of the HRE of MMTV to glucocorticoids in T47D cells shows a similar dependence on DNA topology. These cells have low levels of glucocorticoid receptors and do not respond to dexamethasone (5). Therefore, to study glucocorticoid induction it was necessary to cotransfect the glucocorticoid receptor cDNA in an expression vector, pRSV-GR (19). We have shown that under these conditions, the same negatively supercoiled plasmid pMMTV-TK-CAT-5A responds very efficiently to dexamethasone stimulation (6). Surprisingly, the *Aat*II-linearized plasmid responds similarly (Fig. 1), in clear contrast to the results obtained after progestin induction. In five experiments, the average response of linear plasmids was 100.6% ± 31% of that found with negatively supercoiled plasmids. Again, the linear and supercoiled templates are correctly initiated, as demonstrated by RNase analysis of the transcribed RNA (Fig. 2).

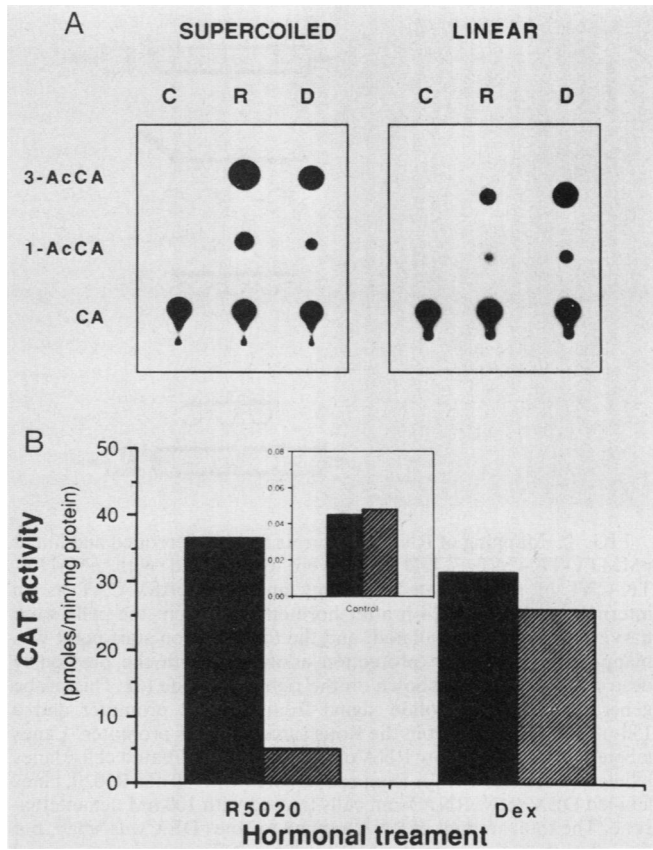

FIG. 1. Hormonal induction of supercoiled and linear pMMTV-TK-CAT in T47D cells. The linear and supercoiled forms of pM MTV-TK-CAT were isolated from agarose gels and transfected into T47D cells, with or without pRSV-GR (see Materials and Methods). After transfection either solvent alone (C), 30 nM R5020 (R), or 100 nM dexamethasone (D) was added to the culture medium, and the CAT activity was determined 40 h later (5). The results of a typical experiment are shown in panel A as an autoradiogram of the thin-layer chromatography plate. (B) Quantitative evaluation of these results. Symbols: ■, results obtained with supercoiled plasmid; ▨, results obtained with linear plasmid. The insert shows the results in the absence of added hormone.

Initial experiments were performed with plasmids linearized with *Aat*II in a region opposite to the location of the HRE, but similar results were obtained when the plasmids were linearized with *Hin*dIII immediately upstream of the HRE (data not shown). To ensure that no recircularization of the linear plasmids could occur in vivo, treated linearized plasmids with dideoxynucleoside triphosphates and the Klenow fragment of DNA polymerase prior to transfection. This leads to the incorporation of dideoxynucleoside triphosphates at the 3′ end of each strand. Plasmids treated in this

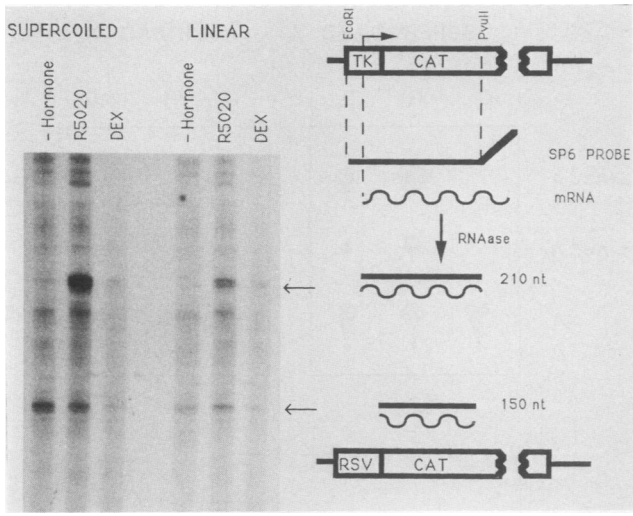

FIG. 2. Mapping of RNA transcripts from supercoiled and linear pMMTV-TK-CAT. T47D cells were transfected with pMMTV-TK-CAT of the indicated topology and with pRSV-CAT as an internal standard. At 24 h after hormone treatment the cells were harvested, RNA was isolated, and the transcription start point was mapped by the RNase protection assay (30) with the previously described pSP6 probe shown on the right-hand side (6). This probe generates a 210-nucleotide signal from the TK promoter and a 150-nucleotide signal from the Rous sarcoma virus promoter. Lanes labeled −Hormone show RNA extracted from untreated cells; lanes labeled R5020 show RNA from cells treated with 30 nM R5020; lanes labeled DEX show RNA from cells treated with 100 nM dexamethasone. The total amount of RNA applied to lanes DEX was small, but the relative intensity of the MMTV-TK-CAT transcript compared with the pRSV-CAT shows correct transcription and dexamethasone induction.

way could not be recircularized in vitro, even when a large excess of DNA ligase was used. With this linear DNA, a normal dexamethasone response was again observed. The low CAT activities detected with linear plasmids in the absence of dexamethasone (Fig. 1B, insert) correspond to faithful transcription, since linearization of the plasmids with *Bam*HI, which cuts between the TK promoter and the CAT gene, completely eliminates CAT activity (data not shown).

Since the effect of glucocorticoids was measured in cells cotransfected with pRSV-GR, whereas the endogenous progesterone receptor was used to study the progestin response,

TABLE 1. Influence of DNA topology on the activity of various promoters and enhancers

Construct and cell line	Hormone	CAT activity[a]	
		Supercoiled DNA	Linear DNA
pRSV-CAT			
T47D	None	20.1	3.2
pMMTV-TK-CAT			
T47D	None	0.04	0.05
T47D	R5020	37.2	5.4
T47D	Dexamethasone	32.3	27.1
XC	None	0.8	0.5
XC	Dexamethasone	6.1	4.8

[a] CAT activity is expressed as picomoles per minute per milligram of protein. Each experiment was performed at least twice and normalized for cotransfected pRSV-lacZ. Although the absolute values varied, the effect of DNA topology was highly reproducible.

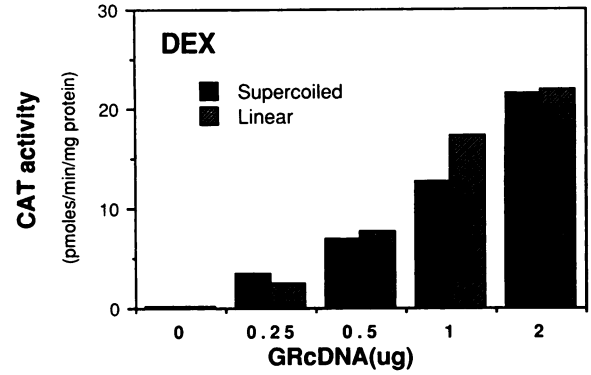

FIG. 3. Influence of glucocorticoid receptor levels on the response to dexamethasone of linear and supercoiled pMMTV-TK-CAT. T47D cells were transfected with a constant amount (1 μg) of linear or supercoiled pMMTV-TK-CAT, together with the amounts of pRSV-GR indicated on the abscissa. The total amount of Rous sarcoma virus-containing plasmid was adjusted to 2 μg with pRSV-lacZ. At 24 h after transfection, the cells were treated with 100 nM dexamethasone and the CAT activity was determined.

we examined the possibility that the lack of influence of DNA topology on the dexamethasone response was due to an overexpression of glucocorticoid receptor. To this end, we first varied the amount of transfected pRSV-GR from 0.25 to 2.0 μg per plate and measured the response to dexamethasone of linear versus supercoiled plasmids. Although the degree of hormonal induction increased considerably with the amount of transfected pRSV-GR, even at low concentrations, no significant difference in hormonal response was detected between supercoiled and linear plasmids (Fig. 3). In addition, when similar experiments were performed with XC cells, a cultured mouse fibroblast cell line containing endogenous glucocorticoid receptor, no difference in response to dexamethasone was observed between plasmids of different topology (Table 1). Thus, the independence of DNA topology seems to be an intrinsic feature of the glucocorticoid response of this plasmid.

Influence of other topological DNA forms on the hormonal response. Having established a difference in response to progestins between negatively supercoiled and linear plasmids, we investigated the behavior of other topological forms of the pMMTV-TK-CAT constructions. None of the topological forms tested significantly influenced the very low level of expression observed in the absence of hormone (data not shown). Circular relaxed DNA behaves similarly to the supercoiled form, whereas the response to R5020 observed with nicked double-stranded plasmids was intermediate between that found for the supercoiled and linear forms (Fig. 4). With respect to the dexamethasone response, there were no significant differences between the different topological forms, suggesting that the amount of transfected DNA was equivalent for DNAs of different topology. The results obtained in the presence of progestins confirmed previous observations of the influence of DNA topology on the activity of enhancer-containing plasmids (28). Thus, a covalently closed circular double-stranded DNA is essential for the detection of the high progestin effect through the MMTV HRE, whereas response to glucocorticoids is relatively independent of the initial topology of the plasmid.

DNA topology does not influence the efficiency of transfection. One possible explanation for the observed effects of topology on the progestin response would be that the efficiency of transfection or the stability of the transfected DNA

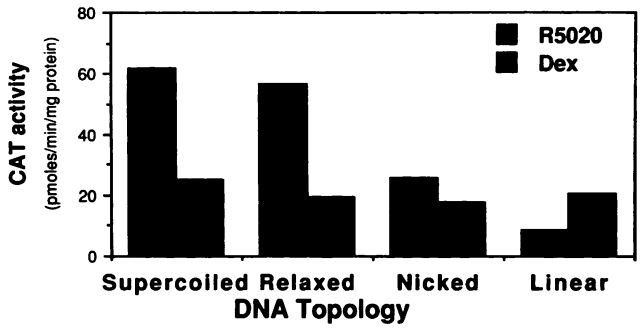

FIG. 4. Influence of DNA topology on the hormonal response of pMMTV-TK-CAT. T47D cells were transfected with different topological forms of pMMTV-TK-CAT prepared as described in Materials and Methods. When dexamethasone was used, pRSV-GR was included in the transfection mixture. After the corresponding hormone treatment, CAT activity was determined and corrected for the internal standard pRSV-lacZ. The values are the average of two independent experiments.

is reduced by linearizing or nicking the plasmids, although the results with dexamethasone induction (Fig. 4) seem to contradict this idea. To directly explore this possibility, we quantified the amount of plasmid DNA recovered from the cell nucleus following transfection with DNAs of different topology (3). In our experiments, hormonal treatment was initiated 24 h after DNA transfection. We selected this time point for DNA quantification, to determine whether the hormone treatment was acting on cells with the same amount of transfected DNA. As an internal reference, supercoiled pUC8 DNA was included in all transfections. The DNA extracted from the purified nuclei was directly electrophoresed on agarose gels or was linearized with *Bam*HI prior to the electrophoresis. The gels were blotted and incubated with radioactive probes that hybridize to the pMMTV-TK-CAT plasmid and the vector pUC8 (Fig. 5a). When corrected for the amount of carrier pUC8, the total amount of pMMTV-TK-CAT recovered from the nuclei 24 h after transfection was similar in cells transfected with negatively supercoiled plasmids or with plasmids linearized and treated with dideoxynucleoside triphosphates. These results show that the topology of the transfected DNA does not significantly influence the efficiency with which the plasmids are incorporated in the nucleus and maintained for at least 24 h after transfection.

We have also analyzed the state of the transfected DNA at different times after transfection of negatively supercoiled plasmids (Fig. 5b). Immediately after transfection, most of the DNA in the cell is either nicked or linearized, but a small proportion (2 to 3% of the recovered DNA) is already found in the negatively supercoiled form. Although the amount of nicked and linear DNA decreases with time after transfection, the amount of supercoiled DNA recovered from the transfected cells remains constant for at least 6 days (Fig. 5b). We also know that the difference in progesterone induction of transcription from linear and supercoiled DNAs is reproducible whether hormone is added immediately after transfection or up to 48 h later (data not shown).

Influence of DNA topology on the affinity of the hormone

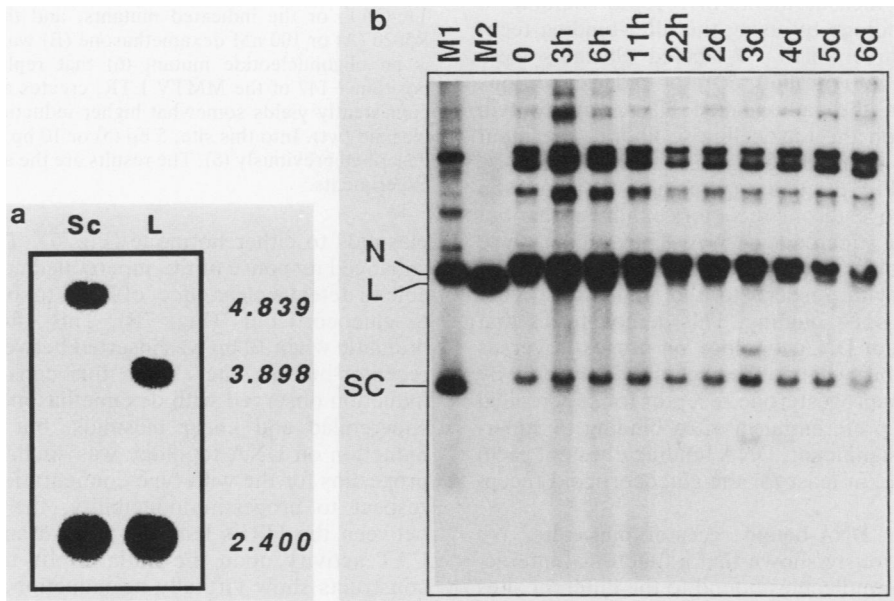

FIG. 5. Quantification of transfected DNA. (a) T47D cells (10^6 cells) were transfected with 2 μg of pMMTV-TK-CAT, either linear (L) or supercoiled (Sc), 2 μg of pUC8 as marker, and 6 μg of carrier calf thymus DNA. At 24 h after transfection, the cells were harvested, washed thoroughly, and treated with DNase I, and the cell nuclei were isolated. The DNA was extracted from the nuclei, linearized with *Bam*HI, and electrophoresed in agarose gels. The gels were blotted and probed with a specific probe to reveal pMMTV-TK-CAT and pUC8. The intact pMMTV-TK-CAT is 4,839 bp. The plasmid linearized with *Aat*II prior to transfection yields a shorter signal (3,898 bp) because the *Bam*HI-*Aat*II fragment is missing. The pUC8 band is 2,400 bp. (b) Fate of negatively supercoiled DNA after transfection. T47D cells (10^6 cells per plate) were transfected with 2 μg of supercoiled pMMTV-TK-CAT DNA. At the indicated times following dimethyl sulfoxide shock, two plates of cells were harvested and treated with DNase I for 30 min at 37°C, and Hirt supernatants were prepared (14). After agarose gel electrophoresis the DNA was transferred to nylon membranes and hybridized with a probe specific for the CAT gene. Lane M1: 1 ng of a mixture of supercoiled and nicked pMMTV-TK-CAT DNA. Lane M2: 1 ng of *Hin*dIII-linearized pMMTV-TK-CAT DNA. The positions of the bands corresponding to supercoiled (SC), nicked (N), and linear (L) DNAs are indicated. The other lanes contain DNA prepared from cells at the indicated times (in hours [h] or days [d]) after transfection.

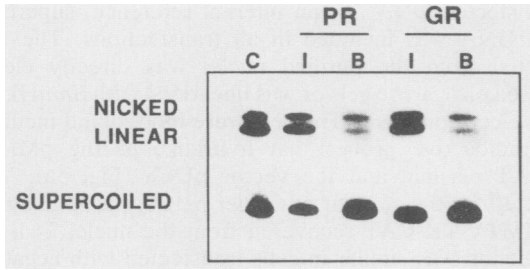

FIG. 6. Influence of DNA topology on DNA binding of glucocorticoid and progesterone receptors. The linear and supercoiled forms of pMMTV-TK-CAT (4 ng each) were labeled to the same specific activity with ^{32}P, as described in Materials and Methods, mixed, and incubated at 0°C for 45 min with partially purified glucocorticoid (6 ng) or progesterone (0.4 ng) receptors. A portion of the input DNA was removed (lanes I), and the rest was filtered through nitrocellulose filters, where the bound DNA was retained (lanes B). The DNA was eluted from the filters and analyzed on 1% agarose gels. Lane C shows the original mixture prior to incubation with the receptors. The nicked DNA originates during handling of the supercoiled form. The DNA bound by either the progesterone receptor (PR) or the glucocorticoid receptor (GR) was enriched in supercoiled DNA molecules and depleted of linear DNA molecules.

receptors for the HRE. A relatively trivial explanation for the observations reported above would be that the progesterone receptor has a higher affinity for the HRE in negatively supercoiled plasmids than in linearized DNA molecules, whereas the glucocorticoid receptor does not distinguish between these two forms of DNA. To address this question, we performed direct measurements of receptor binding to DNA of different topology by using the filter-binding technique. Linear and supercoiled ^{32}P-labeled plasmids were mixed and incubated with purified receptor under conditions where about 20% of the radiolabeled DNA was protein bound and retained on the nitrocellulose filters. The input DNA and that retained on the filters were then analyzed by gel electrophoresis (Fig. 6). The results show that both the glucocorticoid and progesterone receptors exhibit a clear but similar preference for supercoiled DNA. Thus, under these conditions, we cannot detect a difference between the two hormone receptors with respect to the influence of the topological form on DNA binding. This demonstrates that the differential effect of DNA topology on progestin versus glucocorticoid inducibility cannot be accounted for solely by a higher affinity of the progesterone receptor for supercoiled DNA. Although the preferential in vitro binding to supercoiled DNA may be significant, DNA binding does not seem to be the limiting step, at least for the glucocorticoid receptor.

Interaction between DNA-bound receptor molecules. We and others have previously shown that a functional interaction between receptor molecules bound to the different sites within the HRE is required for optimal hormonal response (6, 15). It was therefore tempting to speculate on the possible involvement of DNA topology in mediating this interaction. To investigate this possibility, we used the previously described mutant pLS-147 (6) to analyze the influence of insertions between the two blocks of receptor-binding sites on the topological effect described above. As previously reported (6), an insertion of 5 bp reduces the progestin response and improves the induction by dexamethasone when supercoiled plasmids are used. The same insertion, however, has little influence on the response of linearized

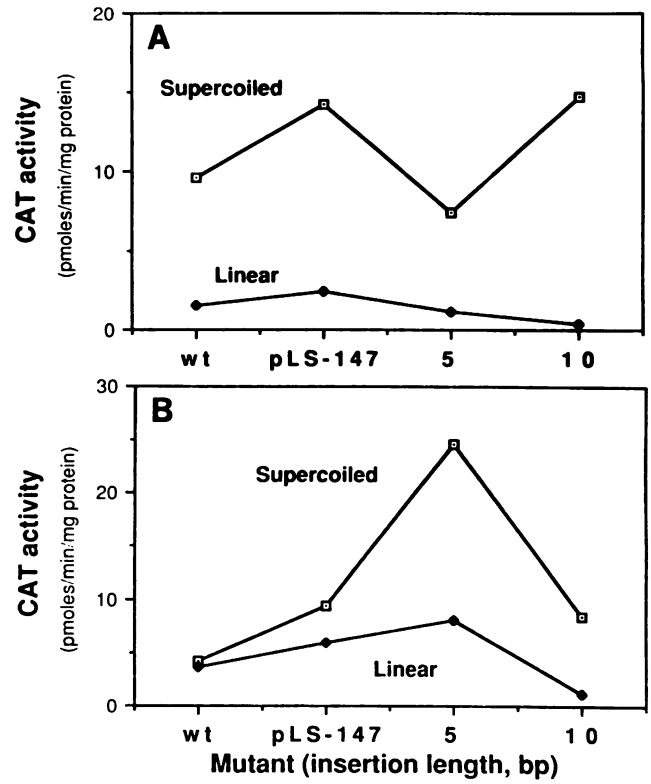

FIG. 7. Influence of insertions between the receptor-binding sites on the response of linear and supercoiled plasmids. T47D cells were transfected with the linear or supercoiled form of pMMTV-TK-CAT, or the indicated mutants, and the response to 30 nM R5020 (A) or 100 nM dexamethasone (B) was determined. pLS-147 is an oligonucleotide mutant (6) that replaces 6 nucleotides at position −147 of the MMTV LTR, creates a unique *ClaI* site, and consistently yields somewhat higher inductions than the wild-type plasmid (wt). Into this site, 5 bp (5) or 10 bp (10) was introduced as described previously (6). The results are the average of two separate experiments.

plasmids to either hormone (Fig. 7). Thus, because of the enhanced response of the supercoiled construct, we are now able to detect a clear effect of DNA topology on the response to glucocorticoids (Fig. 7B). This effect was even more dramatic when 10 bp was inserted between the two blocks of receptor-binding sites. With this construct, the extent of induction observed with dexamethasone decreased for both supercoiled and linear plasmids, but the dependence of induction on DNA topology was similar to that found with progestins for the wild-type configuration of the HRE. With respect to progestin inducibility, the insertion of 10 bp between the HREs led to a more dramatic dependence of CAT activity upon the initial DNA topology. The linear constructs show virtually no induction, whereas the supercoiled mutant is as good as, if not better than, the wild type (Fig. 7A). Thus, one of the functions of DNA supercoiling in these experiments could be to facilitate the interaction between receptor molecules bound to different sites within the HRE. The geometry of the HRE and the relative angular orientation of the receptor-binding sites in the different mutants could determine the extent to which negative supercoiling is required for an efficient interaction between bound receptor molecules.

Interaction with other transcription factors. To test the effect of distance between the HRE and the promoter, we

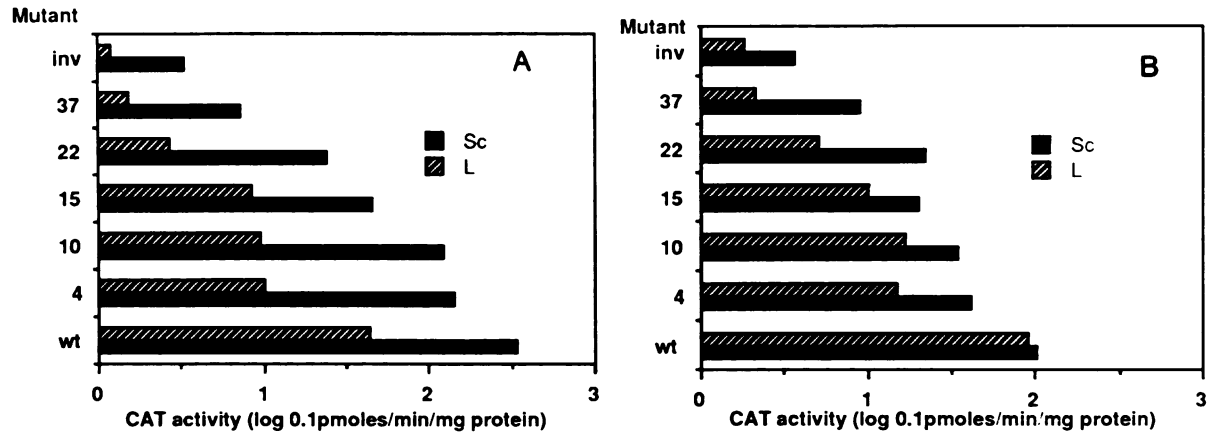

FIG. 8. Influence of insertions between the HRE and the TK promoter on the hormonal response of linear and supercoiled plasmids. T47D cells were transfected with linear and supercoiled forms of the indicated plasmids, and the response to 30 nM R5020 (A) and 100 nM dexamethasone (B) was determined. The construction of plasmids carrying the indicated insertions between the HRE and the TK promoter is described in Materials and Methods. The values represent the average of two independent experiments.

constructed a series of variants of the pMMTV-TK-CAT plasmid in which DNA linkers of different lengths were introduced between the HRE and the TK promoter. Our prediction was that if this distance plays a role, we should be able to confer topological dependence to the glucocorticoid response by increasing the length of the linker DNA. This expectation was fulfilled (Fig. 8B). The insertion of only 4 bp enhanced the topological dependence of the progestin induction and made the dexamethasone response of the supercoiled form two- to threefold better than the response of the linear form (Fig. 8). When 37 bp was introduced between the HRE and the TK promoter, the dependence of the glucocorticoid response on DNA topology was almost complete (note the logarithmic scale). The effect of progestins was also influenced to a greater extent by DNA topology in constructions containing these longer insertions (Fig. 8A). In fact, when the distance between the TK promoter and the HRE (in this case in the reverse orientation) was 300 bp, very little, if any, progestin response was observed with linearized plasmids. Thus, another function of DNA topology could be to facilitate interaction between DNA-bound hormone receptors and other transcription factors bound to the promoter region.

DISCUSSION

The effect of DNA topology depends on the spatial array of protein-binding sites and on the nature of the regulatory proteins. Our results with pRSV-CAT (Table 1) confirm the published observation that in transient transfections, plasmids carrying enhancer elements are better transcribed when covalently closed circular DNA is used (28). Therefore, we expected a similar result with our constructions containing the HRE of MMTV, since this element has been shown to behave as a hormone-dependent enhancer (7). The HRE of MMTV is able to confer to an adjacent promoter the ability to respond to glucocorticoids and progestins (5). Indeed, the receptors for glucocorticoids and progestins bind to the same region of the MMTV long terminal repeat, although the details of the interaction are slightly different (6, 24–26). The results obtained with respect to progesterone induction of different topological forms of pMMTV-TK-CAT confirm our expectations, in that supercoiled and closed-circular relaxed plasmids responded much better than their linear or nicked counterparts. However, when the glucocorticoid response was analyzed with plasmids of different topology, unexpected results were obtained; the induction of pMMTV-TK-CAT by dexamethasone was independent of the initial topology of the transfected plasmid. We know that the natural array of glucocorticoid receptor-binding sites within the MMTV HRE may not be optimal, since the response to dexamethasone can be improved by introducing 5 bp at position −147 between the two blocks of receptor-binding sites (6). When supercoiled plasmids were used, the level of induction by glucocorticoids with this insertion was twice that found with the wild-type configuration, suggesting that the 5-bp insertion leads to a better interaction between glucocorticoid receptor molecules bound to the HRE. In contrast to the results with supercoiled plasmids, the response of the linearized form of this insertion mutant to dexamethasone was not significantly better than the response of the wild type (Fig. 7B). Thus, the 5-bp insertion mutant exhibits a topology-dependent glucocorticoid response. When 10 bp was introduced between the receptor-binding sites, the hormonal response of both linear and supercoiled plasmids was diminished, but the dependence of glucocorticoid induction on DNA topology was more evident. The constructs with a 10-bp insertion also showed a more dramatic effect of DNA topology on progestin induction (Fig. 7A).

We draw two conclusions from these experiments. First, with the wild-type HRE in pMMTV-TK-CAT, two very similar regulatory proteins, namely, the progesterone and the glucocorticoid receptors, exhibit different functional responses to DNA supercoiling. This may reflect the reported differences in the details of the interactions of each hormone receptor with the DNA double helix (6) and underlines the possible functional relevance of these differences. Second, when the two blocks of receptor-binding sites within the HRE are separated by increasing lengths of linker DNA, induction by both hormones becomes more dependent on negative supercoiling. These results suggest that the interaction between DNA-bound receptor molecules needed for optimal hormonal induction is favored when negatively supercoiled plasmids are used for transfection.

A similar effect of DNA topology is observed with mutants that change the distance between the HRE and the pro-

moter. In pMMTV-TK-CAT, the HRE is 30 bp upstream of the more distal binding site for the transcription factor SP1 in the TK promoter (6, 17). With this construction, a clear effect of DNA topology on progestin induction is found, but no significant influence on the glucocorticoid response is detected. However, when the distance between the HRE and the promoter elements is increased, the response of linear DNA molecules to both hormones is impaired more dramatically than that of supercoiled plasmids. With these constructions, a progressive dependence of hormone inducibility on DNA topology becomes more and more obvious, even with glucocorticoids. In fact, when more than 30 bp is introduced between the HRE and the TK promoter, linear plasmids show virtually no response to progestins and the hormonal induction is completely dependent upon the use of negatively supercoiled DNA (Fig. 7). Possibly, these variations in topological dependence reflect differences in the requirements for an interaction between the receptors for the two hormones and other transcription factors. Negative supercoiling could facilitate bending, deformation, and/or looping out of the DNA between distant regulatory sites, and this could favor an efficient interaction between proteins bound to these sites.

Thus, it seems that the functional interaction between DNA-bound glucocorticoid receptor molecules and other transcription factors is more dependent on DNA topology when there is a certain distance between HRE and the other promoter elements. It is conceivable that only in these constructions does the HRE act as a true glucocorticoid-dependent enhancer, as this type of element is defined in terms of its ability to act at a distance.

Possible mechanisms of the topological effect. One possible explanation for the differential dependence of hormonal induction upon DNA topology would be a differential affinity of the corresponding receptor for different topological forms of DNA. If this were the only reason for the observed behavior, we would expect the progesterone receptor to have higher affinity for negative supercoiled than for linear pMMTV-TK-CAT, whereas the glucocorticoid receptor should have similar affinities for both DNA forms. However, the available preliminary data suggest that differential affinity for linear versus supercoiled DNA cannot explain the difference in response to progesterone or glucocorticoids with DNAs of different topology. Under conditions of in vitro binding, both receptors show a similar preference for negatively supercoiled DNA. The observation that an increase in the distance between the intact HRE and the promoter confers topological dependence upon the glucocorticoid response suggests that receptor affinity for the HRE cannot be the only explanation for the observed effect of DNA topology.

We know that the effect of DNA topology on the hormonal response of transfected plasmids is not due simply to differences in the efficiency with which transfected DNA is incorporated into or maintained within the nucleus. We have tried to monitor the fate of transfected DNA as function of time and of initial DNA topology. In agreement with previous results (28), we found that the majority (more than 95%) of plasmids incorporated into the cell are nicked or linearized very rapidly after transfection. However, whereas most of the nicked and the linearized forms decay over a few days, the supercoiled DNA is stably maintained for several days. Similarly, progesterone inducibility is detectable shortly after transfection and remains measurable for at least 4 days. The small proportion of molecules persisting in the supercoiled form are those that remain intact, and these are probably the only ones participating in the observed topological effect. When DNA linearized and treated with dideoxynucleotides is used, no supercoiled DNA is found in the nucleus and the majority of the transfected DNA is degraded shortly after transfection. Since several constructions are normally expressed when transfected as linear molecules in the presence of dexamethasone, this indicates that at least a fraction of the linear DNA can be efficiently transcribed and probably organized into chromatin.

According to previous results, only plasmids carrying an enhancer element are affected by DNA topology (28). However, saturation of the response is achieved with the same amount of plasmids, whether or not they contain an enhancer element, suggesting that the step facilitated by DNA supercoiling is not the binding of proteins to either the promoter or the enhancer itself (28). Rather, it seems that the efficiency with which these bound proteins activate transcription is very sensitive to the initial topological state of the transfected DNA. In agreement with these findings, we know that binding of the receptor to linear DNA fragments containing the HRE is not influenced appreciably by some of the mutations that confer higher dependence on DNA topology. For instance, the introduction of 5 or 10 bp between the receptor-binding sites of the HRE does not change the affinity of linear DNA molecules for the glucocorticoid receptor in any measurable way (6), but it does make dexamethasone induction dependent on the DNA topology. The same may apply for the mutations that change the distance between the HRE and the promoter without altering the sequence of the HRE. Thus, it is probably not the binding of the receptor to the HRE that determines the influence of DNA topology on hormonal response, but some subsequent event needed for transactivation.

The precise array of individual binding sites within the HRE and their relation to the other promoter elements in the wild-type configuration is such that a good response to glucocorticoids is observed after transfection of linear DNA. This could reflect the fact that on the wild-type plasmids the glucocorticoid receptor may act through an alteration in the chromatin structure allowing the transcription factors to enter the promoter (8). Alternatively, the glucocorticoid receptor bound to the wild-type HRE could be located in such a way that its direct interaction with other components of the transcriptional machinery is permitted. The progesterone receptor is known to bind to the MMTV HRE in a somewhat different way, since the limits of the DNase I footprint in the promoter-proximal region of the HRE are different (6). This could explain why the interaction of the progesterone receptor with the factors involved in transcription may be more dependent on DNA topology. When the distance between the HRE and the TK promoter is increased, the glucocorticoid response becomes dependent on DNA topology, and here the HRE is behaving as a glucocorticoid-dependent enhancer element, able to act at a distance. Possibly, when it is acting at a distance, a direct protein-protein interaction between the hormone receptors and components of the transcriptional machinery is essential for optimal induction.

We conclude that the interaction among DNA-bound progesterone receptor molecules needed for optimal hormonal induction and the interaction between receptors and other transcription factors are facilitated by DNA supercoiling. It has been reported that negative supercoiling might decrease the energy required for looping out the DNA between two operator sites bound to the *lac* repressor (16). This could explain, at least in part, the influence of DNA

topology on hormonal response. It is also known that nucleosomes are phased in minichromosomes carrying the MMTV HRE (23), and since this phasing can be reproduced in vitro (20), the nucleotide sequence of the long terminal repeat is probably responsible for the positioning of nucleosomes. It remains to be established whether the organization of plasmid DNA into chromatin is also influenced by the initial topology of the transfected DNAs and how this chromatin structure relates to the observed changes in hormonal response.

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