Possible role of DNA topoisomerase II on transcription of the homeobox gene Hox-2.1 in F9 embryonal carcinoma cells

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ABSTRACT

The Hox-2.1 gene is one of homeobox-containing genes located in the Hox-2 cluster on mouse chromosome 11. In this study, we have examined transcription of the Hox-2.1 gene during differentiation of F9 embryonal carcinoma cells induced by treatment with retinoic acid. The level of Hox-2.1 mRNA increases rapidly after induction of differentiation and then falls. Nuclear run-on experiments demonstrate that the rate of transcription for the Hox-2.1 gene also increases upon differentiation. Treatment of F9 cells with a DNA topoisomerase II inhibitor etoposide (VP-16) during differentiation blocks the accumulation of Hox-2.1 mRNA. Nuclear run-on analyses reveal that etoposide inhibits transcription of the Hox-2.1 gene during F9 cell differentiation. Measurements of the level of Hox-2.1 mRNA after blocking transcription by actinomycin D show that etoposide does not affect stability of the mRNA. These observations indicate that DNA topoisomerase II is involved in the control of Hox-2.1 gene transcription.

INTRODUCTION

The homeobox which encodes a DNA-binding domain of 61 amino acids, was first discovered in genes affecting embryonic development of Drosophila (see Ref. 1 for review). Homeobox-containing genes have been identified and cloned from the genomes of other species, including invertebrates and vertebrates (see Ref. 2 for review). In mouse, more than 30 homeobox genes have been found in four clusters each spanning more than 100 kb of DNA on chromosomes 6, 11, 15, and 2 (the clusters Hox-1, Hox-2, Hox-3, and Hox-4, respectively) (see Ref. 3 for review). They are expressed in specific region at precise time during murine development.

Importantly, a clear relationship is seen among genes in the mouse and Drosophila complexes, based on relative position on the chromosome, sequence homology, and expression pattern along the antero-posterior axis of the embryo. Thus, the genes located in the 5' part are expressed in more posterior regions than genes located in the 3' part of the same cluster (4). Moreover human Hox-2 genes are differentially activated with retinoic acid (RA) in embryonal carcinoma cells in a concentration-dependent manner and in a sequential order which is colinear with their 3' to 5' arrangement in the cluster (5). From these findings one can envision a control mechanism for gene expression, which governs entire chromatin domains of the homeobox gene cluster.

Chromatin DNA of higher eukaryotes appears to be organized into a series of looped domain consisting of about 5 to 100 kb of DNA by anchoring at their basis to the nuclear scaffold or nuclear matrix (6, 7). The domain structure of chromatin could play a role in the control of gene expression by changing the topological state of DNA (8, 9). In consistent with this notion, we have shown that in vitro transcription of eukaryotic genes is affected differently by the degree of DNA supercoiling (10, 11, 12). We have also found that the DNA supercoiling factor can introduce negative supercoils into closed circular DNA in conjunction with eukaryotic DNA topoisomerase II (13). Furthermore, Stief et al. have demonstrated the importance of nuclear scaffold-associated regions (SARS) in expression of genes integrated into chromosomes (14). Interestingly, most of the binding sites for DNA topoisomerase II are associated with the regulatory region of genes and nested in SARS (15, 16, 17). These observations led us to study a role of DNA topoisomerase II in expression of a gene within the homeobox cluster. We demonstrate here that expression of the Hox-2.1 gene is activated at the transcriptional level upon RA-induced differentiation of F9 embryonal carcinoma cells. We show further that transcription of the Hox-2.1 gene is inhibited by treatment of F9 cells with a topoisomerase II inhibitor etoposide.

MATERIALS AND METHODS

Cell culture

F9 embryonal carcinoma cells (18) were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 15% fetal calf serum on gelatinized plastic tissue culture dishes. The cells were induced to differentiate into parietal endoderm cells by addition of 1×10^{-6} M RA and 1×10^{-3} M
dibutyryl cAMP (cAMP) to the medium (19). Etoposide and mAMSA (4'- (9-acridinylamino)-methanesulfon-m-anisidide) were dissolved in dimethyl sulfoxide (DMSO) to 10 mg/ml and added to cultures after appropriate dilution with DMSO. Control samples received the same volume of DMSO instead of the drug. For analysis of stability of mRNAs, cells were treated with 5 

μg actinomycin D per ml of the medium.

RNA isolation and Northern hybridization

Total RNA was isolated from F9 cells essentially as described in Krumlauf et al. (20). The cells were homogenized in an RNA extraction solution (3 M LiCl, 6 M urea, 200 μg/ml heparin sodium salt, 10 mM CH3COONa (pH 5.0), and 0.1% SDS) for 2 min on ice, using a Vertis homogenizer. The homogenate was stored overnight at 0°C, then centrifuged at 9000 g for 20 min at 0°C, and the supernatant was discarded. The RNA pellet was resuspended in 10 mM Tris-Cl (pH 7.4), 1 mM EDTA, and 0.5% SDS, then precipitated again by adding LiCl to 3 M followed by standing at 0°C for more than 3 hr. The RNA was recovered by centrifugation, resuspended in 10 mM Tris-Cl (pH 7.4), 1 mM EDTA, and 0.5% SDS and extracted three times with phenol:chloroform:isoamyl alcohol (25:24:1). Following ethanol precipitation, poly(A)+ RNA was isolated by using oligo(dT)-Latex beads (21).

Ten μg portions of the RNA samples were treated with glyoxal, electrophoresed in a 1% agarose gel and transferred to a Gene Screen Plus membrane (Dupont) in 10× SSC (22). The filter was hybridized in 1% SDS, 1 M NaCl, 10% dextran sulfate, 100 μg/ml of denaturated salmon sperm DNA and 1–5 ng/ml of oligo-prime-d 32P-labeled DNA probe at 68°C for 12–16 hr. The filter was washed twice in 2× SSC at room temperature for 5 min, twice in 2× SSC and 1% SDS at 60°C for 30 min, and finally twice in 0.2× SSC and 1% SDS at 70°C for 1 hr, and then subjected to autoradiography at −80°C with a Dupont-Cronex Lightning Plus intensifying screen. The hybridized probe was stripped off by incubating the filter in an boiling water bath for 5 min beforerehybridization with other probes. The probes used for Northern hybridization were as follows: Hox-2.1 (Hox-2.1 0.66 kb EcoRI/PvuII fragment, see Figure 1); laminin B1 (pLAM (23)); hsp70 (pM1.8, R. Morimoto unpublished); and β-actin (pH659 (24)).

Nuclear isolation and run-on transcription

Nuclei from F9 stem cells and differentiated F9 cells were isolated by a modification of the procedure of Wang et al. (25). Medium was removed from three tissue culture dishes (10 cm in diameter), and the cells were washed twice with ice-cold phosphate-buffered saline (PBS), harvested with a rubber scraper in cold PBS and pelleted by centrifugation at 1000 g for 5 min at 4°C. The pellet was resuspended in 2.5 ml of buffer B (buffer B is the same as buffer A except the sucrose is 0.88 M) and the nuclei were collected by centrifugation at 1000 g for 10 min at 4°C. The nuclear pellet was resuspended in 0.5 ml of buffer C (40% glycerol, 50 mM Tris-Cl (pH 8.0), 5 mM MgCl2, and 0.1 mM EDTA) and pelleted again by centrifugation at 1000 g for 5 min at 4°C. The pellet was resuspended in buffer C to give 2×106 nuclei/ml, divided into 80 μl portions, frozen in liquid N2 and stored at −80°C.

For the run-on transcription assay, an 80 μl portion of nuclei was incubated for 15 min at 30°C in a 200 μl reaction mixture (16% glycerol, 20 mM Tris-Cl (pH 8.0), 2.5 mM MgCl2, 70 mM KCl, 0.5 mM MnCl2, 2.5 mM DTT, 0.8 mM ATP, 0.4 mM UTP and GTP, and 300 μCi [α-32P] CTP (about 650 Ci/mmol). After the reaction, 160 unit/ml of RNase-free DNase I (Pharmacia) were added and the mixture was incubated at 30°C for 5 min, and then equal volume of a solution containing 20 mM Tris-Cl (pH 8.0), 10 mM EDTA, 2% SDS, and 100 μg/ml proteinase K (Boehringer) was added. After incubation at 42°C for 30 min, 1 ml of TE buffer (10 mM Tris-Cl (pH 7.5) and 0.1 mM EDTA) was added and the solution was passed through a 26 gauge needle, then extracted three times with phenol:chloroform:isoamyl alcohol (25:24:1). Aqueous phase was passed over a Sephadex G-50 column (20 ml bed volume) to remove unincorporated [α-32P] CTP. After addition of carrier yeast RNA to 50 μg/ml, RNA was recovered by ethanol precipitation. The precipitate was dissolved in 0.8 ml of DNase buffer (20 mM HEPES (pH 7.9), 5 mM MgCl2 and 1 mM CaCl2) and digested with 200 units/ml of RNase-free DNase I (Pharmacia) for 30 min. After addition of 52 μl 0.25 M EDTA, and 80 μl 10% SDS, the sample was digested with 25 μg/ml of proteinase K at 37°C for 30 min and extracted three times with phenol:chloroform:isoamyl alcohol (25:24:1). RNA was precipitated with ethanol, dissolved in 0.1 ml of TE buffer and purified by passing through a Pharmacia Nick column. The RNA fraction was precipitated with ethanol and dissolved in 60 μl of distilled water. The yield of 32P-labeled RNA from 1.6×107 nuclei was typically 5×106 to 1.5×107 cpm.

Hybridization of 32P-labeled RNA to immobilized DNA

Denatured DNA (10 μg/slot) were absorbed to nitrocellulose filters (Schleicher and Schuell BAA85) by using a slot blot apparatus. Filters were prehybridized for 4–6 hr and hybridized to [32P] RNA for 3 days in 50% formamide, 5×Denhardt’s solution (1×Denhardt’s solution contains 0.02% polyvinyl pyrrolidone, 0.02% Ficoll, and 0.02% bovine serum albumin), 5× SSC, 50 mM NaPO4, 0.1% SDS, and 200 μg/ml denatured salmon sperm DNA at 57°C. The filters were washed twice for 5 min in 2× SSC at room temperature, once in 2× SSC at 60°C for 30 min, twice in 2× SSC, 0.5% SDS at room temperature for 15 min, and in 0.2× SSC, 0.5% SDS at 60°C for 20 min. The filters were washed several times with 2× SSC, and incubated at 37°C in 2× SSC containing 0.2 μg/ml RNase A for 10 min, washed again with 2× SSC and 1% SDS at 37°C for 30 min, and then subjected to autoradiography as described above. DNAs bound on filters were hsp70 (pM1.8, R. Morimoto unpublished); c-fos (pfos-1 (26)); laminin B1 (pLAM (23)); M13

Figure 1. Structure of the Hox-2.1 gene (based upon Ref. 20). Open boxes, the coding regions of Hox-2.1 mRNA; filled box, homeobox sequence; thick line, probe DNA (EcoRI-PvuII fragment) used for Northern hybridization and nuclear run-on assays; E, EcoRI site. The direction of transcription is from the left to the right.
including the hsp70, c-fos, Hox-2.1 and other reference genes, F9 cells. Run-on transcripts were assayed by hybridization to performed on nuclei from undifferentiated and 4 hr RA-induced Hox-2.1 in the rate of transcription, nuclear run-on experiments were of F9 cells.

Rate of Hox-2.1 transcription increases during differentiation of F9 cells

To analyze expression of the Hox-2.1 gene during differentiation of F9 cells, we performed Northern blot analysis of poly(A)+ RNA from cells treated with RA and cAMP for indicated times were separated on an agarose gel and transferred onto a nylon membrane. The blot was hybridized with the 32P-labelled Hox-2.1 probe, and then rehybridized successively with the laminin B1 probe, the hsp70 probe, and the β-actin probe as described in MATERIALS AND METHODS. The exposure times were 50 hr for Hox-2.1, 3 hr for laminin B1, 12 hr for hsp70, and 6 hr for β-actin.

(M13mp18 phage DNA); Hox-2.1 (Hox-2.1 EcoRI/PvuII fragment inserted between the EcoRI/SmaI sites of M13mp18 DNA); and β-actin (pH659 (24)).

RESULTS

Induction of Hox-2.1 transcript upon differentiation of F9 cells

To study a role of DNA topoisomerase II in expression of the Hox-2.1 gene, F9 cells were treated with various concentrations of etoposide for 1 hr at the start of RA-induced differentiation. After the drug was removed, cells were cultured for 3 hr in the presence of RA. Poly(A)+ RNAs were isolated from the cells and analyzed by Northern blot hybridization using the Hox-2.1, the hsp70, or the β-actin probe. The concentration of etoposide is indicated above each lane. The level of Hox-2.1 mRNA in (A) was quantitated by densitometric scanning of the autoradiograms, using the β-actin transcript in each lane as an internal control. The amount in the control sample without etoposide treatment is taken as 100%.

As shown in Figure 3, the hybridization signal for Hox-2.1 in undifferentiated nuclei was barely detectable. However, the signal for Hox-2.1 could be clearly seen after induction with RA. Densitometric analysis reveals that when the Hox-2.1 transcription rates are normalized for hsp70 gene, transcription of the Hox-2.1 gene increases 5-fold upon induction. In contrast to Hox-2.1, none of the reference probes gave elevated hybridization signals in response to RA under these conditions. The transcription rate of the Hox-2.1 gene in cells induced with RA for 8 hr or 16 hr was almost the same level as that in 4 hr-induced cells (data not shown). These results suggest that there is a substantial increase in the transcription rate of the Hox-2.1 gene within early stage of differentiation. This enhancement of transcription appears to explain at least part of the accumulation of Hox-2.1 mRNA.

Inhibition of DNA topoisomerase II affects expression of the Hox-2.1 gene

To study a role of DNA topoisomerase II in expression of the Hox-2.1 gene, F9 cells were treated with etoposide, a specific inhibitor of eukaryotic DNA topoisomerase II (27), during RA-induced differentiation. When etoposide was added at the start of induction and removed after 1 hr, the accumulation of Hox-2.1 mRNA in the absence (−RA/cAMP) or in the presence (4 hr RA/cAMP) of etoposide was severely inhibited (Figure 4A). Under these conditions, there was no detectable change in

Figure 2. Induction of Hox-2.1 mRNA upon differentiation of F9 embryonal carcinoma cells. Poly(A)+ RNAs from cells treated with RA and cAMP for 48 hr in the presence of RA. Poly(A)+ RNAs were isolated from the cells and stained with the Hox-2.1 probe. The level of etoposide was 50 hr for Hox-2.1, 3 hr for laminin B1, 12 hr for hsp70, and 6 hr for β-actin.

Figure 3. Measurement of the rate of Hox-2.1 transcription in F9 cells. Cells were grown in the absence (−RA/cAMP) or in the presence (4 hr RA/cAMP) of etoposide. Nuclei were isolated from the cells and the transcription rates of the Hox-2.1, the hsp70, c-fos, the laminin B1, and the β-actin genes were analyzed by nuclear run-on assay as described in MATERIALS AND METHODS.

Figure 4. Effect of etoposide on expression of the Hox-2.1 gene. (A) F9 cells were treated with various concentrations of etoposide for 1 hr at the start of RA-induced differentiation. After the drug was removed, cells were cultured for 3 hr in the presence of RA. Poly(A)+ RNAs were isolated from the cells and the level of Hox-2.1 mRNA in (A) was quantitated by densitometric scanning of the autoradiograms, using the β-actin transcript in each lane as an internal control. The amount in the control sample without etoposide treatment is taken as 100%.

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Inhibition of DNA topoisomerase II reduces the transcription rate of the Hox-2.1 gene

Considering the transcriptional control of the Hox-2.1 gene during RA-induced differentiation, the observed decrease in the level of Hox-2.1 mRNA by treatment with etoposide is most likely due to inhibition of transcription with the drug. Therefore, we examined the effect of etoposide on the transcription rate of the Hox-2.1 gene by using nuclear run-on assays. When F9 cells were induced with RA for 4 hr and treated with DMSO or 100 μM etoposide, the transcription rate of the Hox-2.1 gene was reduced in the drug-treated sample compared with the control (Figure 5A). The treatment with etoposide scarcely changed the hybridization signals for the hsp70, the c-fos, the laminin B1, and the β-actin genes. The intensities of these bands in this and other two experiments were quantitated by densitometry and the results were normalized for the transcription rate of the hsp70 gene. After etoposide treatment, the level of the Hox-2.1 signal decreased to 26–30% of the control. On the contrary, the hybridization signals for c-fos, laminin B1, and β-actin after etoposide treatment were 83–104%, 75–79%, and 89–116% of the control, respectively. As shown in Figure 5B, essentially the same results were obtained when cells were induced with RA for 8 hr and treated with 100 μM etoposide during the last hour of induction. These results suggest that inhibition of DNA topoisomerase II reduces the transcription rate of the Hox-2.1 gene but scarcely affects transcription of the hsp70, the c-fos, the laminin B1, and the β-actin genes.

Etoposide does not affect stability of Hox-2.1 mRNA

The results of nuclear run-on experiments (Figure 5) show that etoposide inhibits transcription of the Hox-2.1 gene. However, it is possible that etoposide also affects stability of Hox-2.1 mRNA. To test the possibility the level of Hox-2.1 mRNA was measured in RA-induced F9 cells treated with or without etoposide after blocking transcription with actinomycin D. The Northern blot was hybridized with the 32P-labelled Hox-2.1 probe and rehybridized with a probe containing the β-actin gene, which provided an internal control for a stable mRNA (Figure 6A). Quantitation of Hox-2.1 transcripts is shown in Figure 6B, which is based on the results of densitometric scanning of Northern blot and normalized for β-actin mRNA. The amount of Hox-2.1 mRNA in the control cells increased between 7 hr and 10 hr of treatment with RA and cAMP. When 7 hr RA-induced cells were treated with 100 μM etoposide for 1 hr, the level of Hox-2.1 mRNA was gradually decreased. At 10 hr after treatment with RA, the level of Hox-2.1 mRNA from etoposide treated cells was half as much as that from untreated cells. On the other hand, when RNA synthesis was blocked with actinomycin D, there was no difference in the levels of Hox-2.1 mRNA in F9 cells treated with or without etoposide. These results suggest that etoposide inhibits the accumulation of Hox-2.1 mRNA but does not affect stability of preformed Hox-2.1 mRNA.
DISCUSSION

F9 embryonal carcinoma cells differentiate into parietal endoderm by induction with RA and cAMP (19). After 2–3 days of RA treatment, visible morphological changes occur and cells begin to secrete large amounts of basement membrane proteins such as laminin and type IV collagen (25). We show here the transient expression of the Hox-2.1 gene precedes these changes in F9 cells. The accumulation of Hox-2.1 mRNA was detected as early as 4 hr after RA treatment and reached a peak at 24 hr. Similar early expression has been reported for the Hox-1.1 and the Hox-1.3 genes in RA-treated F9 cells (29, 30, 31). Nuclear run-on experiments demonstrate that RA induces the accumulation of Hox-2.1 mRNA at least in part by increasing the transcription rate of the Hox-2.1 gene. Of course, this does not exclude the possibility that the expression of the Hox-2.1 gene is also regulated at the post-transcriptional steps (32).

Etoposide and teniposide (VM-26) interact with eukaryotic DNA topoisomerase II and inhibit it during the breakage—reunion reaction by stabilizing an enzyme–DNA cleavable complex (27). In this study, we have shown the marked inhibition of the accumulation of Hox-2.1 mRNA by treatment of F9 cells with etoposide during RA-induced differentiation. The observed inhibition is not likely due to a general damage in gene expression, because amounts of β-actin and hsp70 mRNA were not changed under these conditions. We consider inhibition of DNA topoisomerase II to be a most likely cause for suppression of Hox-2.1 gene expression, though we cannot rule out the possibility that other than DNA topoisomerase II is the target. In support of this interpretation, the same phenomenon is found by treatment with mAMSA in place of etoposide. mAMSA is known to intercalate DNA and inhibit DNA topoisomerase II by inducing cleavable complex formation between the enzyme and DNA (28).

Nuclear run-on analyses reveal that etoposide inhibits transcription of the Hox-2.1 gene upon F9 cell differentiation. In contrast, total incorporation of [32P] CMP into nuclear RNA and the transcription rate of the hsp70, the c-fos, the laminin B1 or β-actin gene are little affected. Measurements of the level of Hox-2.1 mRNA after blocking transcription with actinomycin D show that etoposide does not affect stability of Hox-2.1 mRNA. These results suggest that the observed inhibition of the accumulation of Hox-2.1 mRNA in etoposide-treated cells is at least partly due to the reduction in synthesis of the mRNA but not due to destabilization of the preformed mRNA. The transcription rate of the Hox-2.1 gene increased 5-fold upon induction with RA and decreased to one fourth by etoposide treatment. How can these apparently small changes in transcription contribute to marked increase or decrease in the level of Hox-2.1 mRNA? Though we have no definite answer for the question, it is possible that Hox-2.1 transcript being rapidly turned over in stem cells becomes stable upon induction with RA and that inhibition of DNA topoisomerase II switches it back to the unstable state. The results presented here indicate that DNA topoisomerase II is required for expression of the Hox-2.1 gene but it is not essential for that of the hsp70, the c-fos, the laminin B1, and the β-actin genes. Recently, Dunaway has shown that inhibition of DNA topoisomerase II by teniposide does not affect expression of the rRNA gene and the thymidine kinase gene in Xenopus oocytes (33). This is also consistent with the idea that DNA topoisomerase II is required for expression of only a limited number of genes.

There are several possibilities to explain the observed inhibitory effect of etoposide on transcription of the Hox-2.1 gene. The first explanation is that the movement of the transcribing RNA polymerase is physically blocked by the formation of topoisomerase II–DNA cleavable complexes on the Hox-2.1 coding region. This possibility seems less likely, because no cleavable complex was found within the transcribed region of the Hox-2.1 gene when F9 cells were treated with etoposide or mAMSA (unpublished results). The second explanation is that DNA topoisomerase II is required for relaxation of the supercoils generated by tracking of RNA polymerases along the helical path of duplex DNA (34). This possibility also seems to be remote because only transcription of the Hox-2.1 gene was inhibited by treatment with etoposide among five genes tested while transcription-driven supercoiling of DNA should occur on any genes. The twin supercoiling domain model predicts that the degree of supercoiling is high if two oppositely oriented genes are being transcribed on the same template (35). However, all the Hox genes in the Hox-2 cluster are transcribed into the same orientation (4). The third possibility is that the promoter activity of the Hox-2.1 gene is strongly influenced by superhelical density of the template, which is controlled by DNA topoisomerase II. It has been shown that the rate of transcription of various genes in vitro responds differently to changes in template superhelicity (10, 11, 12), and that eukaryotic DNA topoisomerase II can introduce negative supercoils into DNA in conjunction with supercoiling factor (13). The last explanation is that DNA topoisomerase II is necessary to decondensate the chromatin structure of a looped domain which include Hox-2.1 gene prior to transcription and maintain the decondensed state during transcription. The Hox-2.1 gene is one of the Hox-2 cluster genes, which are differentially activated along the antero-posterior axis of the mouse central nervous system: the genes that lie in the more 5' region of the cluster have more posterior restriction to their pattern of expression (4). It is possible that the Hox-2 cluster is organized into a looped domain of chromatin and that DNA topoisomerase II regulates the conformation of the chromatin domain from the basis of the loop. Further studies are necessary to establish the role of DNA topoisomerase II in the transcriptional regulation of the Hox-2.1 gene.

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