Erratum

Complex pattern of alternative splicing generates unusual diversity in the leader sequence of the chicken link protein mRNA

by F. Deák, E. Barta, S. Mestric, M. Biesold and I. Kiss


The publishers wish to apologize for the incorrect legends accompanying Figures 1, 2 and 3 of this article. Supplementary data not intended for publication was inadvertently included. The correct legends are published below.

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**Fig. 1.** Nucleotide sequence of the 5' end of the chicken LP gene. Positions are given from the translation start site within exon 2, not including intron 1 (nucleotides in lower case letters). Nucleotide sequences represented by cDNA clones are indicated by solid lines. All sequences were determined from both directions. TATA and CCAAT motifs are boxed. Homopurine and homopyrimidine tracts longer than 30 nt are denoted by double and single broken lines, respectively. Arrows point to the major transcription start sites. Nucleotides identical with the human sequence (31) are marked with asterisks above the sequence.

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**Fig. 2.** Composite representation of the transcription initiation sites determined by various techniques. The TATA-like sequence is boxed. Thickness of the horizontal arrows below the sequence reflects the relative frequency of initiation.
Fig. 3. Mapping of transcription start sites by T4 DNA polymerase. The diagram to the left depicts the experimental strategy. An M13 DNA carrying the RNA-complementary strand of exon 1 and the promoter region was used as template. Solid bar with asterisk represents the $^{32}$P end-labeled PA primer, broken arrow indicates the primer extension products. Lanes A, C, G and T, dideoxy sequencing ladders of the same template DNA from unlabeled PA primer in the presence of $[^{35}]$SdATP. Lane 1, 4 µg poly(A)$^+$ RNA was hybridized to the template before primer extension. Lane 2, primer extension in the absence of hybridized mRNA. The numbers at the right margin of the autoradiogram denote the nucleotide positions. That part of the autoradiogram which carries the extension product at position $-487$ is shown after prolonged exposure, in an inlet to the right.

Hypervariable polymorphism in the APOC3 gene

by S.Bhattacharya, T.M.E.Wilson, A.P.Wojciechowski, C.P.Volpe and J.Scott


An incorrect figure was inadvertently supplied with this paper. The correct figure is reprinted below.

Frequency was estimated in 30 unrelated Caucasian English individuals, in whom 24 alleles could be distinguished. Only one of the 30 individuals was homozygous. The heterozygosity index was 0.95, and the polymorphic information content 0.94. Further alleles e.g. 317 nt have also been noted. A constant band measuring 340 nt was seen in 4 of 30 individuals.
Editorial

Deposition of atomic coordinates and other data for crystal structures

Notes for Authors

1. Nucleic Acids Research now requires that atomic coordinates for crystal structures be deposited in a database prior to manuscript submission.

2. The appropriate databases are as follows:
   - Nucleosides and nucleotides and other small molecules: Cambridge Crystallographic Data Centre (CCDC). Please note that Nucleic Acids Research does not normally accept papers on small molecules unless the results are of relevance to the structure of nucleic acids.
   - Proteins, polypeptides etc: Protein Data Bank (PDB).
   - Oligonucleotides: Deposition may be made to either CCDC or to PDB. The two databases have agreed that atomic coordinates and thermal parameters deposited to one database will automatically be transferred to the other. It is also strongly recommended that structure factors be deposited simultaneously. In this case it is recommended that deposition be made to PDB since CCDC does not currently store structure factors. For oligonucleotides longer than 25 residues, deposition must be made to PDB.

3. Delayed Release: The databases will make available atomic coordinates following publication of the article. Delayed release stipulations by authors will not be permitted. Other data is not subject to this requirement at this time.

4. Procedure for deposition at CCDC: Submission should be made on a CCDC Data Deposition Form which can be obtained from CCDC, University Chemical Laboratory, Lensfield Road, Cambridge, CB2 1EW, UK (tel +44 (0) 223 336408, FAX + 44 (0) 223 312288, EMAIL DGW1@UK.AC.CAM.PHX). Information should be deposited preferably by EMAIL or on floppy disk or alternatively as hard copy (typed listing or computer printout). A copy of the CCDC Data Deposition Form must be submitted to Nucleic Acids Research together with the manuscript. If the paper is not accepted for publication, the editor will inform CCDC who will hold the data and await further communication from the author. Each paper must carry the following statement: 'The author has deposited atomic coordinates for this structure with the Cambridge Crystallographic Data Centre, University Chemical Laboratory, Lensfield Road, Cambridge, CB2 1EW, UK'.

5. Procedure for deposition at PDB: Submission should be made by FTP, EMAIL, or on a magnetic medium, accompanied by a PDB Data Deposition Form and copies of all relevant articles. Deposition forms and further details can be obtained from Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, New York 11973, USA (tel +1 516 282 3629, FAX +1 516 282 5815, EMAIL PDB@BNLCHM.BITNET). In addition to atomic coordinates, thermal parameters and structure factors are also accepted and their deposition is strongly urged. The PDB will acknowledge receipt by issuing an entry identification code. This identification code will be included in published papers and must be forwarded to the editor by the authors. Failure to do so may result in delay in publication.

Please note corrected telephone number for the Protein Data Bank.
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 topoisomerse 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 
\( \mu \)g 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 
\( \mu \)g/ml heparin sodium salt, 10 mM CH\(_3\)COONa (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 
\( \mu \)g 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 X SSC (22). The filter was hybridized in 1% SDS, 1 M NaCl, 10% dextran sulfate, 100 
\( \mu \)g/ml of denatured salmon sperm DNA and 1-5 
\( \mu \)g/ml of oligo-primed 32P-labeled DNA probe at 68°C for 12-16 hr. The filter was washed twice in 2 x SSC at room temperature for 5 min, twice in 2 x SSC and 1% SDS at 60°C for 30 min, and finally twice in 0.2 x 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 before rehybridization 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 \( \beta \)-actin (pHE659 (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 600 g for 5 min at 4°C. The cell pellets were resuspended in 2.5 ml of buffer A, vortexed, and homogenized by 5 strokes in a Kontes tissue homogenizer using the B pestle. The homogenate was then layered over 2.5 ml 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 MgCl\(_2\), 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 x 10\(^6\) nuclei/ml, divided into 80 
\( \mu \)l portions, frozen in liquid N\(_2\) and stored at -80°C.

For the run-on transcription assay, an 80 
\( \mu \)l portion of nuclei was incubated for 15 min at 30°C in a 200 
\( \mu \)l reaction mixture (16% glycerol, 20 mM Tris-Cl (pH 8.0), 2.5 mM MgCl\(_2\), 70 mM KCl, 0.5 mM MnCl\(_2\), 2.5 mM DTT, 0.8 mM ATP, 0.4 mM UTP and GTP, and 300 
\( \mu \)M [\( \alpha \)-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 
\( \mu \)g/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 [\( \alpha \)-32P] CTP. After addition of carrier yeast RNA to 50 
\( \mu \)g/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 MgCl\(_2\) and 1 mM CaCl\(_2\)) and digested with 200 units/ml of RNase-free DNase I (Pharmacia) for 30 min. After addition of 52 
\( \mu \)l 0.25 M EDTA, and 80 
\( \mu \)l 10% SDS, the sample was digested with 25 
\( \mu \)g/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 
\( \mu \)l of distilled water. The yield of 32P-labeled RNA from 1.6 x 10\(^6\) nuclei was typically 5 x 10\(^8\) to 1.5 x 10\(^7\) cpm.

**Hybridization of 32P-labeled RNA to immobilized DNA**

Denatured DNA (10 
\( \mu \)g/slot) were absorbed to nitrocellulose filters (Schleicher and Schuell BA85) 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 x Denhardt’s solution (1 x Denhardt’s solution contains 0.02% polyvinyl pyrrolidone, 0.02% Ficoll, and 0.02% bovine serum albumin), 5 x SSC, 50 mM NaPO\(_4\), 0.1% SDS, and 200 
\( \mu \)g/ml denatured salmon sperm DNA at 57°C. The filters were washed twice for 5 min in 2 x SSC at room temperature, once in 2 x SSC at 60°C for 30 min, twice in 2 x SSC, 0.5% SDS at room temperature for 15 min, and in 0.2 x SSC, 0.5% SDS at 60°C for 20 min. The filters were washed several times with 2 x SSC, and incubated at 37°C in 2 x SSC containing 0.2 
\( \mu \)g/ml RNase A for 10 min, washed again with 2 x 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 (pjes-1 (26)); laminin B1 (pLAM (23)); M13
results

Induction of Hox-2.1 transcript upon 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 prepared from cells in the undifferentiated stage or cells cultured for various lengths of time in medium containing RA and cAMP. The Northern 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 EcoRl/PvuII fragment inserted between the EcoRl/SmaI sites of M13mp18 DNA); and β-actin (pH659 (24)).

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

To investigate whether the observed accumulation of Hox-2.1 mRNA during differentiation of F9 cells is due to an increase in the rate of transcription, nuclear run-on experiments were performed on nuclei from undifferentiated and 4 hr RA-induced F9 cells. Run-on transcripts were assayed by hybridization to filter-bound DNA probes for Hox-2.1 and other reference genes, including the hsp70, c-fos, the laminin B1, and the β-actin genes.

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 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.

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 during 4 hr of induction was severely inhibited (Figure 4A). Under these conditions, there was no detectable change in
cell morphology. Rehybridization of the same membrane with a probe containing the hsp70 or the \( \beta \)-actin gene revealed that the levels of these mRNAs were not affected by addition of etoposide.

Densitometry of Hox-2.1 and \( \beta \)-actin mRNAs was carried out and the level of Hox-2.1 mRNA was normalized for that of \( \beta \)-actin mRNA. As shown in Figure 4B, 40 \( \mu \)M or higher concentration of etoposide decreased the amounts of Hox-2.1 mRNA to less than 10% of control. Moreover, the accumulation of Hox-2.1 mRNA during 4 hr of induction was also inhibited if mAMSA, another inhibitor of DNA topoisomerase II (28), was added during the first hour of induction, while the level of hsp70 or \( \beta \)-actin mRNA was left unchanged (data not shown). These results suggest that inhibition of DNA topoisomerase II suppresses expression of the Hox-2.1 gene but does not affect expression of the hsp70 and the \( \beta \)-actin genes.

**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 \( \mu \)M etoposide during the first hour of induction, no significant difference in the amounts of \([32P]\) CMP incorporated into nuclear RNA was observed between the control and etoposide-treated samples (data not shown). However, the hybridization signal for Hox-2.1 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 \( \beta \)-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 \( \beta \)-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 \( \mu \)M 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 \( \beta \)-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 \( \beta \)-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 \( \beta \)-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 \( \mu \)M 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.

**Figure 5.** Etoposide inhibits transcription of the Hox-2.1 gene. (A) F9 cells were treated without (−Etoposide) or with 100 \( \mu \)M etoposide (+Etoposide) for 1 hr at the start of RA-induced differentiation. After removal of the drug, cells were cultured for 3 hr in the presence of RA. Nuclei were isolated from the cells and the transcription rate of the Hox-2.1, the hsp70, the c-fos, the laminin B1, and the \( \beta \)-actin genes were analyzed by nuclear run-on assays as described in MATERIALS AND METHODS. (B) F9 cells induced with RA for 8 hr were treated without (−Etoposide) or with 100 \( \mu \)M etoposide (+Etoposide) during the last hour of induction. Nuclei were isolated and subjected to nuclear run-on assays as above.

**Figure 6.** Effect of etoposide on stability of Hox-2.1 mRNA. (A) 7 hr RA-induced F9 cells were divided into four parts. A portion was continued to culture in the normal medium (control cells). The second portion was treated with 100 \( \mu \)M etoposide for 1 hr and then the drug removed and the culture continued. Another two portions received 5 \( \mu \)g/ml of actinomycin D and treated with or without etoposide as above. Poly(A)\(^{+}\) RNAs were isolated from the cells at indicated times and analyzed by Northern blot hybridization using Hox-2.1 or \( \beta \)-actin probe. (B) The level of Hox-2.1 mRNA in (A) was quantitated by densitometric scanning of the autoradiograms, using the \( \beta \)-actin transcript in each lane as a control. The amount in 7 hr RA-induced cells is taken as 100%. Control (O), treated with etoposide (●), treated with actinomycin D (△), treated with actinomycin D and etoposide (▲).
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 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 decondense 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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REFERENCES