INTRODUCTION

Ewing’s sarcoma is a highly aggressive bone and soft tissue tumor of adolescents. In a subset of patients, a specific t(21;22) chromosomal translocation involving EWS and the ETS related gene, ERG, generates an EWS/ERG fusion protein (1,2). CADO-ES1 is an Ewing’s sarcoma cell line that can differentiate into neural and mesenchymal cell lineages (3,4). Here we show that CADO-ES1 cells express EWS/ERG, and a range of splicing variants of the α2(XI) collagen gene (COL11A2).

Since COL11A2 is linked to neural and mesenchymal cell phenotypes (5,6), and ERG transcription factor is involved in chondrogenesis (7), we investigated whether ERG and EWS/ERG participate in regulating COL11A2 expression.

MATERIALS AND METHODS

Cell culture and RT-PCR. CADO-ES1 cells were cultured in RPMI 1640 medium with 10% FBS, 5% CO$_2$ at 37°C (2). RT-PCR and DNA sequencing of EWS/ERG and COL11A2 were performed as described (2,3). Plasmids. The cDNAs for ERG, EWS/ERG, and their mutants were cloned into pSG5-FL with an N-terminal Flag epitope. The DNA sequences for mouse Col11a2 (5,6) and human COL11A2 promoter regions (9) were cloned into pGL3 Basic luciferase reporter vector (Promega).

Transfection and luciferase assay. For promoter analysis, CADO-ES1 cells were transfected with pGL3 reporter and pRL-SV40 internal control using DOTAP (Roche). Forty-eight hours later, cells were assayed for luciferase activity using Dual Luciferase Reporter Assay System (Promega). NIH3T3 cells were similarly transfected and assayed with pSG5-FL effector, pGL3 reporter and pRL-SV40 control. In one experiment, cells untreated or treated with 100 ng/ml or 500 ng/ml of Trichostatin A (Sigma) were lysed and assayed for luciferase 18 hours after treatment.

Electrophoretic mobility shift assay (EMSA). EMSA was performed on CADO-ES1 nuclear extract as described previously (10) using COL11A2 promoter sequences.

Immunoprecipitation and Western blotting. Lysates from CADO-ES1 cells and transfected NIH3T3 cells were immunoprecipitated with anti RNA Pol II antibody (Ab), anti ERG Ab, or control IgGs, as described (11,12). Immunoprecipitates were separated by SDS-PAGE and the proteins detected with anti ERG Ab or anti Flag Ab using an ECL system (Amersham).

RESULTS AND DISCUSSION

RT-PCR and DNA sequencing analysis showed that CADO-ES1 cells expressed EWS/ERG type 1 fusion transcripts and also abnormally spliced COL11A2 transcripts. The mouse Col11a2 promoter sequence from −742 bp to +380 bp, from the transcriptional start site, was activated in CADO-ES1 cells when transiently transfected and assayed for luciferase activity. Deletion analysis identified a minimum promoter region, which contains two ETS DNA binding sites, with a 39 bp deletion resulting in a 3.5-fold induction (Figure 1). The human COL11A2 promoter also showed similar activity. Deletion analysis identified an essential promoter region, which contains two predicted ERG binding sites and three SP1 sites. Mutations of the COL11A2 promoter indicated that multiple sub-regions regulated its activity. EMSA detected several nuclear proteins that interacted with the COL11A2 promoter. To investigate whether ERG and EWS/ERG differentially affected the COL11A2 promoter, transfection experiments were carried out in NIH3T3 cells, in which the endogenous Col11a2 promoter is inactive. While EWS/ERG induced Col11a2/COL11A2 promoter-driven luciferase activity in transfected NIH 3T3 cells, EWS/ERG with a defective ETS DNA binding domain, or intact ERG did not.

We recently reported that the N-terminal half of ERG interacts with ESET, a novel histone H3-specific methyltransferase (13) and that ESET can recruit histone deacetylase (unpublished data). We speculated that intact ERG is silenced through its association with the ESET-histone deacetylase complex while EWS/ERG is not because EWS replaces the N-terminal domain. After treatment with the histone deacetylase inhibitor Trichostatin A, ERG activated the COL11A2 promoter as effectively as did EWS/ERG. We also reported previously that TLS/ERG binds to RNA polymerase II, while intact ERG does not (11). Immunoprecipitation and Western blot analysis of lysates of CADO-ES1 cells or transfected NIH3T3 cells also showed that EWS/ERG, not ERG, was bound to RNA Pol II. Together, these findings indicate that upregulated expression and abnormal mRNA splicing of COL11A2 in CADO-ES1 cells may be a direct result of RNA Pol II recruitment and failed histone modification through the nuclear activity of EWS/ERG.

CONCLUSION

These results provide evidence for a differential regulation of transcription by EWS/ERG and ERG. The expression of COL11A2 in CADO-ES1 cells may be related to the suspected neural/mesenchymal origin of Ewing’s sarcoma. It seems likely that other genes with promoter properties in common with COL11A2 are also differentially regulated by EWS/ERG and ERG. The implications we believe are fundamental to an understanding of Ewing’s tumor pathobiology.