A specific t(21;22) chromosomal translocation creates the chimeric EWS/ERG gene in some cases of Ewing's sarcoma. In the resultant EWS/ERG fusion protein, the N-terminal part of the ETS family protein ERG is replaced by the N terminus of the RNA-binding protein EWS. We found that both the EWS/ERG and COL11A2 genes are expressed in the Ewing's sarcoma cell line, CADO-ES1. To investigate a potential role for EWS/ERG in COL11A2 gene expression, we characterized the COL11A2 promoter and tested the ability of wild-type ERG and EWS/ERG sarcoma fusion protein to transactivate COL11A2 promoter using a luciferase assay. We found that expression of EWS/ERG, but not wild-type ERG, transactivated the COL11A2 promoter and that this transactivation required not only the N-terminal region of EWS but also an intact DNA-binding domain from ERG. Electrophoretic mobility shift assay using COL11A2 promoter sequence showed involvement of EWS/ERG in the formation of DNA-protein complexes, and chromatin immunoprecipitation assay revealed direct interaction between COL11A2 promoter and EWS/ERG fusion protein in vivo. EWS/ERG, but not wild-type ERG, bound to RNA polymerase II. Treatment of cells with the histone deacetylase inhibitor trichostatin A enabled ERG to transactivate the COL11A2 promoter, therefore abolishing the differential effects of EWS/ERG and ERG. Taken together, these findings indicate that the COL11A2 gene is regulated both by potential ERG association with a histone deacetylase complex and by direct EWS/ERG recruitment of RNA polymerase II.

Ewing's sarcoma is a highly aggressive bone and soft tissue tumor that occurs in adolescents and is of unknown tissue origin (1). Most Ewing's sarcomas exhibit a specific t(11;22) chromosomal translocation that results in the fusion of EWS to the ETS family member Fli-1. In a subset of patients, a specific t(11;22) translocation creates a specific EWS/ERG fusion protein and wild-type ERG.

Received for publication, January 7, 2003, and in revised form, January 27, 2003 Published, JBC Papers in Press, January 28, 2003, DOI 10.1074/jbc.M300164200

Yoshiito Matsui‡, Howard A. Chansky‡, Fariba Barahmand-Pour‡, Anna Zielinska-Kwiatkowska‡, Noriyuki Tsumaki§, Akira Myoui§, Hideki Yoshikawa§, Liu Yang‡†, and David R. Eyre‡‡

From the §Department of Orthopedics and Sports Medicine, University of Washington School of Medicine, Seattle, Washington 98195-6500 and the ¶Department of Orthopedic Surgery, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

© 2003 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.

This paper is available on line at http://www.jbc.org

The abbreviations used are: Pol, polymerase; CREB, cAMP-response element-binding protein; RT-PCR, reverse transcription-PCR; EDB domain, ETS DNA-binding domain; ESET, ERG-associated protein with a SET domain; TSA, trichostatin A; TRT, tandem repeat of the tct trinucleotides.
**Differential COL11A2 Regulation by EWS/ERG and ERG**

**Plasmid Constructs**—Various lengths of the DNA sequences for mouse Col11A2 promoter regions (9, 10) were cloned into Mlu/XhoI sites of pGL3 basic luciferase reporter vector (Promega, Madison, WI) (Fig. 1c). To characterize the minimum human COL11A2 promoter sequence between −149 and +27 bp (H149 construct in Figs. 1c and 2a), deletion constructs were created as H68 (−84 to +27 bp), H68 (−68 to +27 bp), and H40 (−40 to +27 bp). In addition, mutations were introduced into human COL11A2 promoter constructs as follows: mTRET (mutated tandem repeat of the tcc trinucleotides) contains a 4-base mutation from tctctc to tATCA located between −130 and −124 bp of the H149 construct; dH84 (deletion mutant of H84 construct) contains sequences of −84 to −59 bp and −40 to +27 bp; mSp1-u (mutated upstream Sp1 site) contains an 8-base mutation from gggggggg to cccccc between −82 and −62 bp; mSp1-d (mutated downstream Sp1 site) contains a 5-base change mutation from gggg to TTTcTT located between −50 and −45 bp of the H149 construct; mSp1-u/d contains mutations at both upstream and downstream Sp1 sites of the H149 construct.

The cDNAs for ERG and EWS/ERG were cloned into the pSG5-FL expression vector (18). The resultant proteins are FLAG epitope-tagged downstream Sp1 sites of the H149 construct. mSp1-u/d contains mutations at both upstream and downstream Sp1 sites of the H149 construct.

**Immunoprecipitation and Western Blotting**—CADO-ES1 cells or transfected NIH3T3 cells in a 10-cm dish were lysed with 1.2 ml of buffer A (10 mM Tris, pH 7.4, 100 mM NaCl, 2.5 mM MgCl2, 0.5% Triton X-100, 10 mM DTT) supplemented with protease inhibitor mixture and phosphatase inhibitor mixture (Sigma). The supernatant was also used in the assay. 32P-Labeled DNA probe was incubated with 5 µl of fresh cell lysate for 60 min at 4 °C. The complex was then incubated with 0.2 ml of Protein A/G PLUS-agarose (Santa Cruz Biotechnology) and 0.2 ml of 1X SDS sample buffer was added to the agarose beads. The protein samples were denatured for 5 min at 95 °C, separated by SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and subjected to Western blotting with the C-20 rabbit polyclonal anti-Pol II antibody (Amersham Biosciences). Protein bands were visualized with the ECL Western blotting analysis system (Amersham Biosciences).

**RESULTS**

**CADO-ES1 Cells Express Both COL11A2 and EWS/ERG**—Our previous RT-PCR analysis revealed that the expression and splicing patterns of COL11A2 are linked to a chondrocyte phenotype in osteochondrogenic tumors (15). The Ewing’s sarcoma cell line CADO-ES1 also expressed COL11A2 transcripts, but the splicing pattern of COL11A2 transcripts in Ewing’s sarcoma cells differed from that in chondrocytes (Fig. 1a, lanes 1 and 2). Specifically, most COL11A2 transcripts in CADO-ES1 cells retained combinations of alternative exons 6–8, whereas those in chondrocytes did not. Further analysis by RT-PCR and DNA sequencing showed that CADO-ES1 cells also express EWS/ERG fusion transcript of type I (Fig. 1, a, third panel from top, and b).

Although there are no reported effects of EWS/ERG fusion protein on collagen gene expression, a function for wild-type ERG protein in chondrogenesis is well documented (14, 21). Chondrogenesis can be monitored by the induction of cartilage-specific collagen genes (e.g., type II, IX, and XI collagens) (13). We hypothesized that ERG and/or EWS/ERG are directly involved in the expression of collagen genes and, in particular, the COL11A2 gene in CADO-ES1 Ewing’s sarcoma cells. It has been reported that the mouse Col11a2 promoter sequence, −742 to +380 bp from the transcriptional start site, is sufficient to drive reporter gene expression (11), and multiple subregions between −742 and −453 bp are known to be involved in cartilage- and neural tissue-specific promoter activities (12).

The mouse Col11a2 gene is located next to the retinoic acid receptor β (Rxrb) gene in a head-to-tail arrangement (Fig. 1c, top line) (11). Human COL11A2 and RXRB are arranged similarly. The intergenic DNA sequences of mice and humans are highly homologous (Fig. 1c, bottom line) (22). The transcriptional start site in mouse corresponds to −327 bp from the human transcriptional start site (Fig. 1c) (11, 23).

In order to identify the promoter sequence responsible for COL11A2 expression in CADO-ES1 cells, a range of pGL3 luciferase reporter plasmids containing various lengths of mouse Col11a2 promoter sequences were generated (Fig. 1c).

The mouse Col11a2 promoter sequence from −742 to +380 bp was activated when transfected into CADO-ES1 cells (Fig. 1, c and d, construct −742). Interestingly, in CADO-ES1 cells, the mouse promoter constructs −530 and −500 containing either
the cartilage- or the neural tissue-specific cis-elements (Fig. 1, top line) showed similar activity to the /H11002 construct containing only the constitutive promoter sequence between /H11002 and /H11001 (Fig. 1, c and d) (12). Further deletion analysis identified a necessary minimum sequence between /H11001 and /H11001 of the mouse promoter, which corresponds to /H11002 to /H11001 bp of the human COL11A2 promoter (Fig. 1, c and d, compare construct /H11001 and construct /H11001). The evolutionally conserved human COL11A2 promoter sequence between /H11001 and /H11001 bp was therefore cloned into the luciferase reporter vector and was equivalent in activity to the intact mouse Col11a2 promoter when transfected into CADO-ES1 cells (Fig. 1, c and d, construct /H11001).

The Human COL11A2 Promoter Contains Multiple Regulatory Subregions—Inspection of the DNA sequence between /H11002 and /H11002 of the human COL11A2 promoter revealed tcc tcc, a tandem repeat of the tcc trinucleotides (TRT) (24), as well as at least two /gggggc Sp1 binding sites (Fig. 2a, construct /H11001). To test them for functional activity, a series of deletion and mutation constructs were generated for transfection (Fig. 2a). Deletion between /H11002 and /H11002 bp, containing the TRT sequence decreased promoter activity by 50% (Fig. 2, construct /H11001). Mutation in the TRT sequence, from tcc tcc to tAActAA (uppercase letters indicate mutated nucleotides), also decreased promoter activity (Fig. 2, construct mTRT), suggesting that the TRT sequence is involved in the expression of COL11A2 in CADO-ES1 cells. Deletion between /H11002 and /H11002 bp, containing the TRT sequence and the upstream Sp1 site (Sp1-u) decreased promoter activity by 70% (Fig. 2, construct H68). Deletion of the TRT sequence and the downstream Sp1 site (Sp1-d) also decreased promoter activity by 70% (Fig. 2, construct dH84). Deleting the DNA sequence between /H11002 and /H11002 bp nearly abolished the promoter activity (Fig. 2, construct H40), thus confirming the importance of the TRT sequence and Sp1 sites. Involvement of both Sp1 sites was further supported by mutations from /gggggc to TTTcTT for the upstream Sp1 site and from /ggggg to TTTcTT for the downstream Sp1 site. Whereas mutating just one of the two Sp1 sites resulted in a 50% decrease (Fig. 2, construct mSp1-u and mSp1-d), mutations at both Sp1 sites led to a 75% decrease in promoter activity (Fig. 2, construct mSp1-u/d). Together, these results indicate that in CADO-ES1 cells, the TRT site, and the Sp1 sites coordinately regulate COL11A2 promoter activity.

Collagen gene regulation by Sp1 has been documented extensively (25), whereas protein interactions with the TRT se-
quence have not been thoroughly investigated. To detect potential interactions between CADO-ES1 nuclear proteins and the TRT sequences within human COL11A2 promoter, an electrophoretic mobility shift assay was performed using a 40-bp DNA oligonucleotide matching −149 to −110 bp from the COL11A2 transcriptional start site (Fig. 2a). CADO-ES1 nuclear extract and the TRT sequence formed DNA-protein complexes designated as C1 and C2 (Fig. 3a, compare lanes 1 and 2). The addition of wild-type TRT competitor prevented formation of the C1 and C2 complexes (Fig. 3a, lane 3). Furthermore, both C1 and C2 complex formation was eliminated by a specific anti-ERG antibody that recognizes the C-terminal portion of EWS/ERG fusion protein but not by a control IgG (Fig. 3b, lanes 7 and 8). Thus, EWS/ERG and/or ERG in CADO-ES1 nuclear extract appear to specifically interact with the TRT sequence within the human COL11A2 promoter.

We next performed a chromatin immunoprecipitation assay to determine whether EWS/ERG fusion protein selectively binds to COL11A2 promoter in CADO-ES1 cells. Acetylated histone molecules are known to be enriched in areas near actively transcribed genes such as GAPDH (20). HeLa cells do not express COL11A2; therefore, the COL11A2 promoter region was not co-precipitated with an anti-Ac-H3 antibody in the chromatin immunoprecipitation assay (Fig. 3c, compare lanes 9 and 10). In CADO-ES1 cells, the COL11A2 gene is active, and its promoter sequence becomes cross-linked to acetylated histone H3 after treatment with formaldehyde (Fig. 3c, compare lanes 11 and 12). Interestingly, COL11A2 promoter sequence was enriched in immunocomplexes using an antibody that recognizes the N-terminal domain of EWS/ERG, and this enrichment appeared to be specific as GAPDH DNA was absent from the same immunocomplexes (Fig. 3c, lane 13). These results suggest that EWS/ERG fusion protein interacts with COL11A2 promoter in vivo.

**EWS/ERG, but Not ERG, Activates the COL11A2 Promoter in NIH3T3 Cells**—To investigate whether ERG and EWS/ERG can transactivate the COL11A2 promoter, ERG and EWS/ERG expression constructs were generated for co-transfection with the COL11A2 promoter reporter construct. Our initial experiments showed minimal effects of ERG or EWS/ERG on COL11A2 promoter activity when transfected into CADO-ES1 cells (data not shown). We suspected that this might be caused by abundant endogenous EWS/ERG in CADO-ES1 cells; therefore, the experiments were repeated with NIH3T3 cells that do not express EWS/ERG or Colla2 (26). Whereas wild-type ERG protein had no effect on COL11A2 promoter activity, the EWS/ERG sarcoma fusion protein potently transactivated human COL11A2 promoter construct H149 (Fig. 4). To investigate whether the transactivation was dependent on the ability of EWS/ERG to bind DNA, we constructed EWS/ERG mutants that lacked the EDB domain or had either of two EWS/ERG point mutants (W351R or R367L) within the EDB domain (Fig. 4b). The W351R mutation of EWS/ERG is equivalent to the W321R mutation of Fli-1, an ETS protein with high sequence homology to ERG. Both point mutations are known to eliminate the DNA binding activity of Fli-1 and ERG (27, 28). When co-transfected into NIH3T3 cells with the COL11A2 promoter construct H149, neither the EWS/ERG Δ EDB nor the two point mutations were able to transactivate the promoter (Fig. 4b), suggesting that a functional EDB domain within EWS/ERG is critical for activation of the COL11A2 promoter.

**EWS/ERG, but Not ERG, Is Physically Associated with RNA Pol II, and EWS/ERG Is Less Susceptible than ERG to Repression by Histone Deacetylases**—Although both ERG and EWS/ERG have identical DNA binding domains, our findings show that only EWS/ERG is able to transactivate the COL11A2 promoter construct. It is important to understand the mechanism underlying this intriguing finding, since EWS is known to fuse with the DNA-binding domains of a variety of transcription factors in different sarcomas. Part of the explanation for the differential action may lie in the properties of the EWS component of the EWS/ERG fusion protein. Both wild-type EWS and EWS/Fli-1 sarcoma fusion protein have been reported to associate with RNA Pol II (5). If the N-terminal region of EWS is indeed responsible for binding to RNA Pol II, then EWS/ERG should likewise interact with RNA Pol II. To test for this, CADO-ES1 cell lysate was treated with an anti-RNA Pol II antibody. Endogenous EWS/ERG but not ERG was co-precipitated with the RNA-Pol II, whereas a control mouse IgG did not immunoprecipitate either protein (Fig. 5a). With lysate from transfected NIH3T3 cells, the same anti-RNA Pol II antibody pulled down the FLAG-tagged EWS/ERG but not ERG (Fig. 5b). Together, these results show that EWS/ERG, but not ERG, binds to RNA Pol II.

Another property contributing to this dichotomous behavior could be a loss of function from the absence of the N-terminal ERG domain in the EWS/ERG sarcoma fusion protein. Our recent cloning and analysis of an ERG-associated protein with a SET domain (ESET) revealed that ESET protein acts as a histone H3-specific methyltransferase (29). Histone methyl-
transfersases usually function in complexes with histone deacetylases to silence gene expression (30–32), and ESET has been shown to recruit histone deacetylases as well as mSin3 transcriptional corepressors (33). Since EWS/ERG lacks the N-terminal domain of ERG responsible for binding to ESET, we suspected that ESET-associated histone deacetylases could suppress ERG-mediated transactivation, whereas these same histone deacetylases would have no such effect on EWS/ERG.

Our approach in testing this hypothesis is based upon prior studies of histone deacetylases using reporter constructs (34–36). Transiently transfected plasmid DNA does not usually assume a native chromatin structure, but published observations indicate that histone molecules do associate with plasmid DNA to form nucleosome-like particles (37, 38). A shift toward increased acetylation of these nucleosome-like particles can result in a more open structure that is accessible to the transcriptional machinery. In fact, inhibitors of histone deacetylases have been reported to similarly up-regulate promoter constructs in both transiently and stably transfected cells (39, 40).

We co-transfected NIH3T3 cells in duplicate plates with the COL11A2 promoter construct H149 plus either ERG or EWS/ERG expression plasmid and then treated cells in one plate with TSA, a well known inhibitor of histone deacetylases (41). Transfected cells in the other plate were not treated with TSA; therefore, they were used as a control to calculate -fold derepression by TSA. Whereas the ability of wild-type ERG protein to transactivate the COL11A2 promoter was significantly increased in the presence of TSA, transactivation by EWS/ERG fusion protein was not as profoundly influenced by TSA-mediated inhibition of histone deacetylases (Fig. 6). The differential effects of EWS/ERG and ERG on COL11A2 promoter thus appear to result in part from their differing abilities to recruit RNA Pol II and to associate with histone deacetylases.

**DISCUSSION**

Our findings indicate that ERG and the sarcoma fusion protein EWS/ERG differentially regulate expression of the COL11A2 gene. Although both wild-type ERG and the EWS/ERG fusion protein share the same DNA-binding domain and are expected to bind to the same promoter sequence, the human COL11A2 promoter construct is transactivated by EWS/ERG but not by ERG. Recent reports have shown that EWS fusion proteins can inhibit expression of several genes (3, 42). In contrast, our results together with a recent report on induction of the Id2 gene by EWS-ETS (43) reveal that an EWS fusion protein is able to activate an authentic promoter that is not activated by its wild-type partner. We also show that mutations within the tandem repeat of tetrancleotides of the COL11A2 promoter sequence or within the ETS-DNA binding domain of EWS/ERG protein interfere with COL11A2 activation. Furthermore, electrophoretic mobility shift and chromatin immunoprecipitation assays confirm the direct interaction of EWS/ERG and/or ERG with the COL11A2 promoter. Thus, transactivation is at least partially mediated by interaction between the COL11A2 promoter and the DNA binding domain of EWS/ERG.

In this study, we also investigated why EWS/ERG, but not ERG, transactivates COL11A2. Our study points to at least two potential mechanisms. First, since inhibition of histone deacetylation significantly enhanced the effects of ERG on COL11A2 promoter activity, transactivation by ERG appears to be epigenetically suppressed. This is very similar to the proposed mechanism for Fli-1 inhibition of COL11A2 transcription (25). Interestingly, recent studies have shown that a murine Kruppel-associated box-zinc finger protein represses Col11a2 promoter activity (44), and human ESET (also called SETD81) is known to bind to the universal, obligatory corepressor of Kruppel-associated box-zinc finger proteins (45). On the other hand, a recent study showed that the N-terminal region of wild-type EWS interacts with CREB-binding protein that has histone acetyltransferase activity (6). It may be that EWS/ERG also associates with CREB-binding protein and promotes derepression of transcription via acetylation of histones bound to its target promoters. Second, as evidenced by immunoprecipitation experiments, EWS/ERG fusion protein recruits RNA Pol II in vivo, whereas wild-type ERG does not. By fusing the N terminus of EWS to the DNA-binding domain of ERG, the EWS/ERG oncoprotein is able to recruit RNA Pol II directly to the site of transcription, and this could serve to bypass suppression by histone deacetylase complexes. These findings therefore provide new insights into the mechanisms underlying collagen gene expression in general and COL11A2.
In addition to transcriptional deregulation, EWS sarcoma fusion proteins are reported to interfere with basic cellular processes such as RNA splicing. Unlike wild-type EWS protein, the C-terminal domains of EWS fusion products are unable to recruit serine-arginine splicing factors (5), which may partly explain the abnormal splicing patterns in Ewing’s sarcoma cells. The finding that EWS/ERG binds RNA Pol II also suggests a coupled mechanism for stimulated gene expression and altered RNA splicing. Since gene transcription and RNA splicing are physically linked via the RNA Pol II holoenzyme, EWS/ERG has the potential to deregulate gene activation, epigenetic gene repression, and RNA splicing. All of these processes are integral to cell growth and differentiation.

Expression of \textit{COL11A2} in CADO-ES1 cells supports a neural/mesenchymal origin of Ewing’s sarcoma (10, 46). Interestingly, the regulatory elements of the \textit{COL11A2} active in CADO-ES1 cells differ from those active in cells of cartilage and neural lineages (12, 47). The \textit{COL11A2} promoter shares many features with certain housekeeping promoters (23), such as the presence of G/C-rich sequences, the absence of a TATA box, and multiple transcription start sites. Similar to the findings here with the \textit{COL11A2} promoter, the TATA-less, GC-rich promoters of the human heparanase-1 and the mouse thymidylate synthase genes are synergistically regulated by ETS family transcription factors and the Sp1 protein (48, 49). It is likely that other genes with promoter properties in common with \textit{COL11A2} are also differentially regulated by EWS/ERG and ERG, and the implications, we believe, are therefore fundamental to an understanding of Ewing’s tumor pathobiology.

**Acknowledgments**—We thank Drs. Ken Kodama for the CADO-ES1 cells, Mirka M. Vuoristo for cosmid clone 505-1, Ituro Inoue for a PAC clone containing the human \textit{COL11A2} region, and Kristin Rosler for technical assistance in the chromatin immunoprecipitation assay.

**REFERENCES**

1. Ginsberg, J. P., de Alava, E., Ladanyi, M., Wexler, L. H., Kovar, H., Paulussen, M., Zoubek, A., Dockhorn-Dworniczak, B., Juergens, H., Wunder, J. S., Andruolo, I. L., Malik, R., Sorensen, P. H. B., Womer, R. B., and Barr, F. G. (1999) \textit{J. Clin. Oncol.} 17, 1809–1814

2. Sorensen, P. H. B., Lessnick, S. L., Lopez-Terrado, D., Liu, X. F., Triche, T. J., and Denny, C. T. (1994) \textit{Nat. Genet.} 6, 146–151

3. Im, Y.-H., Kim, H. T., Lee, C., Poulin, D., Welldorf, S., Sorensen, P. H. B., Denny, C. T., and Kim, S.-J. (2000) \textit{Cancer Res.} 60, 1536–1540

4. Ohno, T., Ouchida, M., Lee, L., Gatalica, Z., Ras, V. N., and Reddy, E. S. P.
