Brief Genetics Report

Identification of *Dep-1*, a New Gene Regulated by the Transcription Factor Pax-3, as a Marker for Altered Embryonic Gene Expression During Diabetic Pregnancy

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The causes of diabetic embryopathy, in which congenital malformations arise in the early developing embryo of a pregnant mother with preexisting diabetes, are poorly understood. Previously, we reported that malformations may be caused by abnormal expression of important regulatory genes in the embryo (1). In particular, using a mouse model of diabetic embryopathy, a threefold increase in neural tube defects (NTDs) appears to result from reduced expression of *Pax-3* (1). *Pax-3* encodes a transcription factor that is required for neural tube, neural crest, and somitic mesoderm development. Thus, altered expression of genes that are regulated as part of a Pax-3–dependent process may result from diabetic pregnancy, and altered expression of some of these genes may contribute to defective embryonic development.

There are very few identified genes that are transcriptionally regulated as part of a Pax-3–dependent process (2–5), none of which, with the exception of the gene encoding N-CAM (6,7), which may be regulated posttranscriptionally by Pax-3, participate in neural tube development. To further characterize the molecular defects that occur as a consequence of altered expression of *Pax-3* during diabetic embryopathy, it is necessary to identify additional markers of Pax-3–deficient transcriptional responses. Toward this end, we used differential polymerase chain reaction (PCR) display (8), using RNA obtained from day-9.5 embryos of diabetic and nondiabetic mice and from embryos derived from the *Splotch* strain of mice, which carry loss of function *Pax-3* alleles. A 237–base pair (bp) cDNA that corresponds to a transcript that is reduced in embryos of diabetic mice and in homozygous *Splotch* embryos was identified by this method. Sequence analysis of this cDNA suggested that it had not previously been identified. Because we identified this gene from embryos of diabetic mice that are prone to embryopathy, we named it *Dep-1* (Diabetic Embryopathy-1).

To more rigorously evaluate whether Dep-1 RNA was reduced in embryos of diabetic mice during the time in which neural tube formation occurs, a sensitive semiquantitative reverse transcription–polymerase chain reaction (SQ-RT-PCR) assay (1) was performed, using Dep-1–specific primers. (The experimental method is available in an on-line appendix at www.diabetes.org/diabetes/appendix.htm.) Although no differences in expression of *Dep-1* between embryos of diabetic or nondiabetic mice were detected either on embryonic day 8.5, the day on which *Pax-3* expression and neural tube formation begins (9), or on day 10.5, when fusion of the neural tube in most embryos is complete, there was a twofold reduction in *Dep-1* expression in embryos of diabetic mice on day 9.5, when neural tube formation is in progress (Fig. 1*A*). As previously reported (1), *Pax-3* expression was reduced threefold in embryos of diabetic mice on days 8.5 and 9.5 of gestation. Expression of a control gene, *36B4*, was not reduced at any time examined. This demonstrates that decreased expression of *Dep-1* follows reduced expression of *Pax-3* and occurs when formation of the neural tube is in progress, but is not observed when neural tube fusion is complete.

Evidence that the reduction in *Dep-1* resulted from reduced expression of *Pax-3* was obtained using the mouse mutant *Splotch* (*Sp*). The *Splotch* allele contains a single point mutation in a splice acceptor site within the Pax-3 transcript, preventing the production of any functional Pax-3 protein (10–12). Thus, the molecular defect in *Splotch* embryos is due solely to the absence of Pax-3 and the consequent misexpression of genes that are regulated as part of a Pax-3–dependent process. Dep-1 RNA was reduced approximately twofold in *Sp/Sp* embryos compared with pooled wild-type (wt) and *Sp/+* embryos (Fig. 1*B*). Because the only genetic difference between *Sp/Sp* and pooled wt and *Sp/+* embryos is the presence of functional Pax-3 protein, this demonstrates that *Dep-1* must be regulated, directly or indirectly, as part of a Pax-3–dependent process.

Further evidence that expression of *Dep-1* is positively regulated by Pax-3 was obtained using P19 embryonal carcinoma cells. *Pax-3* expression can be induced in P19 cells by treatment with retinoic acid (13). As shown in Fig. 1*C*, the

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bp, base pair; NTD, neural tube defect; PCR, polymerase chain reaction; *Sp*, *Splotch*; SQ-RT-PCR, semiquantitative reverse transcription–polymerase chain reaction; wt, wild-type.

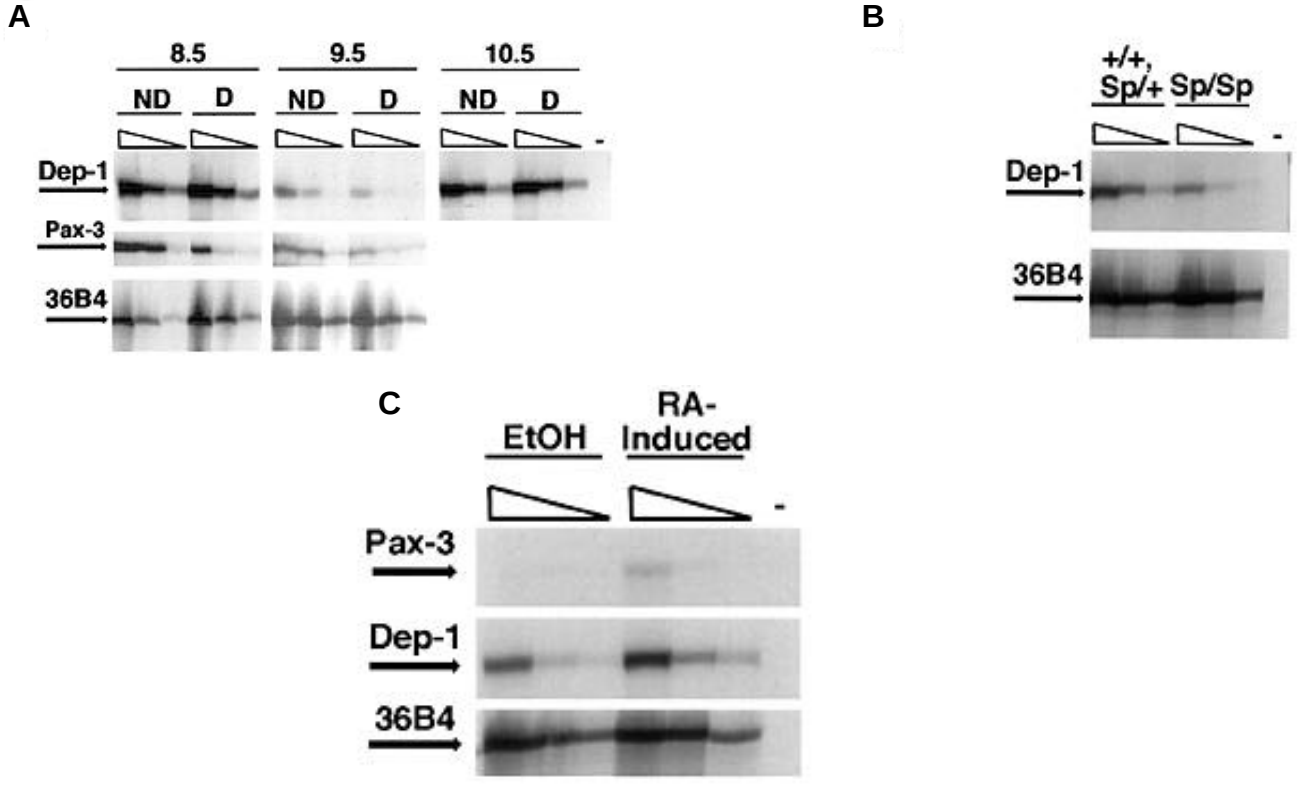

FIG. 1. *A*: **SQ-RT-PCR using RNA from embryos of nondiabetic (ND) or diabetic (D) mice on days 8.5, 9.5, or 10.5 of gestation. Serial dilutions of reverse transcriptase reaction products were used for PCR with Dep-1 or Pax-3 primers (using from 1:4 to 1:64 dilutions) or 36B4 primers (using from 1:64 to 1:4,096 dilutions).** *B*: **SQ-RT-PCR using RNA obtained from day-9.5 embryos of heterozygous *Splotch* (*Sp*/+) matings. Embryos displaying NTDs (*Sp*/*Sp*) were separated from the remaining wt (+/+) and *Sp*/+ embryos. –, H_2O added to PCR in place of reverse transcriptase reaction products as a PCR control. The specific forward and reverse primers for Dep-1 cDNA were CAGCTATTACACATGAACATGC and CCTCCACAATTTTTCAAATGCC, respectively, which produce a 237-bp cDNA fragment. The specific forward and reverse primers for 36B4 (18), which was used as a control, were CGACCTGGAAGTCCAACTAC and TGATAGCCTTGCGCATCATG, respectively, which produce a 177-bp cDNA fragment. The specific primers for Pax-3, which produce a 145-bp cDNA fragment have been published (1).** *C*: **SQ-RT-PCR of P19 cells treated either with ethanol alone (EtOH) or retinoic acid dissolved in ethanol (RA). PCR using Pax-3 and Dep-1 primers was performed with serial dilutions of reverse transcriptase reactions ranging from 1:1 to 1:16, and PCR using 36B4 primers was performed with serial dilutions ranging from 1:16 to 1:256. –, H_2O added to PCR in place of reverse transcriptase reaction products.**

induction of Pax-3 mRNA by retinoic acid was accompanied by a twofold increase in Dep-1 RNA. This result is consistent with the notion that *Dep-1* is controlled by Pax-3 in a cell culture model of neuroectodermal cells as well as in whole embryos.

The ability to detect Dep-1 RNA in uninduced P19 cells suggested that *Dep-1* may be a constitutively expressed gene that is further activated upon induction of *Pax-3*. In support of this, we found that *Dep-1* is expressed as early as day 6.5 in whole embryos (2 days before the onset of *Pax-3* expression) in all tissues of day-13.5 embryos, including, but not limited to, neural tube derivatives, and in all adult mouse tissues that we examined (data available at www.diabetes.org/diabetes/appendix.htm). This indicates that *Dep-1* is regulated in part by factors3 that are not tissue specific, and in part by tissue-specific factors that accentuate its expression. It is possible that variable amounts of Dep-1 transcript are needed by different cells and that tissue-specific factors, such as Pax-3, regulate the accumulation of Dep-1 transcript according to the needs of the cell. A variation on this theme is that the transcriptional activity of *Dep-1* may be modulated by cell-specific derepression that serves to inactivate ubiquitously expressed repressors of the *Dep-1* gene and that Pax-3 participates in this process. Because of the ubiquitous expression of *Dep-1* in the whole embryo, it is surprising that a difference in *Dep-1* expression was detected at all by SQ-RT-PCR. Thus, the twofold difference in *Dep-1* expression in whole embryos appears to be an underestimate of the *Dep-1* response to Pax-3; because the majority of cells in the day-9.5 embryo are not *Pax-3* expressing, the magnitude of the *Dep-1* response in Pax-3-responsive cells must be vastly greater than twofold.

The 237 bp Dep-1 partial cDNA was used as a probe to obtain a 3.3-kb cDNA from a day-11.5 mouse embryo cDNA library, the sequence of which can be accessed by GenBank number AF032310. Comparison of both strands of the 3.3-kb Dep-1 cDNA to protein and DNA databases indicated that this cDNA was novel. There are several short open reading frames, the longest of which is only 96 amino acids in length, suggesting that *Dep-1* could encode a small protein. However, because of the existence of multiple translation termination codons in each reading frame, it is intriguing to consider that *Dep-1* belongs to the emerging class of genes that encode untranslated regulatory RNA molecules. Some untranslated transcripts, such as Xist, which is responsible for X chromosome inactivation (14), and H19, which is responsible for the genomic imprinting of two paternally expressed genes, *Ins-2* (murine insulin-2) and *Igf-2* (insulin-like growth factor-2) (15,16), are essential for embryonic development.

We have recently reported that expression of *cdc46*, a gene whose product is involved in the regulation of DNA synthesis, is negatively regulated by Pax-3 in embryos of diabetic mice (17). Thus, *Dep-1* is the second gene, and the first newly identified gene, that has been found to be regulated by Pax-3 and to be associated with diabetic embryopathy. This indicates that the reduction in *Pax-3* expression during maternal diabetes causes molecular disturbances in expression of Pax-3 target genes. Unlike *cdc46*, whose increased expression could be observed as soon as *Pax-3* expression was reduced, the defect in *Dep-1* expression was delayed by 1 day. This suggests that *cdc46* may be directly regulated by Pax-3, while *Dep-1* may be an indirect downstream target. Although our experiments using P19 cells demonstrate that a *Dep-1* response can occur in the same cells that express *Pax-3*, it is certainly possible that the reduced expression of *Dep-1* in embryos may not be limited to *Pax-3*–expressing cells. For example, Pax-3 could regulate expression of a secreted factor that controls *Dep-1* by a paracrine mechanism. The failure to observe reduced expression of *Dep-1* on day 10.5, when neural tube formation is complete but when *Pax-3* expression is still reduced in somites and limb buds (S.A.P., M.R.L., unpublished observations), could indicate that *Dep-1* transcription is only affected by Pax-3 before the stage of development that coincides with neural tube fusion or that there are redundant pathways in somites and limb buds that compensate for Pax-3 insufficiency with regard to *Dep-1* expression.

Whether and how *Dep-1* regulates neural tube formation, or any other biological processes, awaits further investigation. The results presented here demonstrate that the reduction in *Pax-3* expression in embryos of diabetic mice is sufficient to affect at least one additional downstream molecular process besides inhibition of expression of *cdc46* (17). Thus, the use of *Dep-1* as a marker for Pax-3–dependent transcription can be used to assist in the investigation of molecular processes that are operational during diabetic embryopathy.

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Author Queries (please see Q in margin and underlined text)

Q1: <<Au: Correct that the protein encoded by the *Pax-3* gene is also called Pax-3? And that this protein is what is being referred to in the construction “Pax-3–dependent”?>
Q2: <<Au: Correct that “*Splotch*” has been italicized in the name of this mouse strain because it is a gene name?>
Q3: <<Au: Please indicate which authors are from each institution.>
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