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Original Article

Expression of the E2F family of transcription factors during murine development

JUDITH C. KUSEK, ROBERT M. GREENE, PAUL NUGENT and M. MICHELE PISANO*

Department of Molecular, Cellular and Craniofacial Biology, ULSD, University of Louisville Birth Defects Center, Louisville, KY 40292, USA

ABSTRACT The E2F family of transcription factors plays a crucial role in the control of cell cycle progression and regulation of cellular proliferation, both processes fundamental to mammalian development. In the present study, we have examined the levels of expression of the six currently identified E2F proteins in murine embryos/fetuses as a function of gestational age, compared the expression of these six proteins in selected developing and adult tissues, and examined E2F expression in the embryonic murine palate, a tissue in which perturbation of proliferation is associated with induction of cleft palate. Our results indicate that: 1) multiple forms of individual E2F family members are present in embryonic, fetal and adult cells/tissues; 2) each of the six E2Fs is expressed in a tissue specific manner in both adult and embryonic/fetal organs; 3) certain forms of individual E2F family members are preferentially detected in adult tissues, whereas others are preferentially expressed in embryonic/fetal tissues; 4) expression of the various E2Fs and their isoforms follows distinct temporal patterns during murine gestation; and 5) individual E2F family members also exhibit differential patterns of temporal expression during murine palatogenesis.

KEY WORDS: *E2F, craniofacial, embryogenesis, palate, transcription factor*

Introduction

Mammalian ontogenesis is characterized by a precisely regulated sequence of events contingent upon the basic developmental processes of morphogenesis, cell differentiation and growth by cell proliferation. Hence, a precise balance between positive and negative regulation of cell division is critical to normal mammalian development. The mammalian cell cycle functions to integrate the myriad of stimulatory and inhibitory growth- and differentiation-related signals that impinge upon an embryonic cell, making the cell cycle a critical determinant of the developmental status of tissues and organs. In recent years, the E2F family of transcription factors has emerged as a key regulator of the cell cycle, poised to control both proliferation and differentiation by virtue of its ability to regulate both progression *through* the cell cycle, and stasis *within* the G_0 and G_1 phases of the cell cycle, a prerequisite for differentiation. These opposing actions of the E2Fs are accomplished via two distinct mechanisms: 1) transcriptional activation during late G_1 and early S phases of the cell cycle, and 2) active silencing of gene expression during G_0 and early G_1. The balance between these two functional states appears to depend on the levels of the various E2Fs in the nucleus and on their ability to form complexes with members of the retinoblastoma (Rb) family of tumor suppressor proteins (for reviews see: Bernards, 1997; Dyson, 1998; Helin, 1998; Nevins, 1998; Lavia and Jansen-Durr, 1999).

When present in high concentrations, uncomplexed E2F-1 is a potent activator of transcription. Microinjection or overexpression of E2F-1 induces DNA synthesis driving cells past the restriction point and into the S phase of the cell cycle (Johnson *et al.*, 1993; Dobrowolski *et al.*, 1994; Qin *et al.*, 1994; DeGregori *et al.*, 1995; Kowalik *et al.*, 1995; Lukas *et al.*, 1996). Moreover, overexpression of E2F-1 can overcome the growth inhibitory effects of TGFβ (Schwarz *et al.*, 1995), serum deprivation (Johnson *et al.*, 1993), and p21 overexpression (Leone *et al.*, 1999). Dominant negative mutants of E2F-1 or its heterodimeric partner, DP-1, produce the opposite effect, arresting cells in G_1 (Wu *et al.*, 1996; Fan and Bertino, 1997). This ability to regulate progression through the cell cycle appears to be a function of transcriptional activation of various arrays of genes involved in DNA replication and/or expression of critical components of the cell cycle machinery (for review see, Slansky and Farnham, 1996).

The E2F proteins readily complex with the hypophosphorylated forms of the Rb family of proteins (pRb, p107 and p130). As depicted in Figure 1, complex formation converts E2F into an active silencer of transcription. The E2F component of these complexes

Abbreviations used in this paper: aa, amino acid; GD, gestational day; kDa, kilodalton; PVDF, polyvinylidene difluoride; PBS, phosphate-buffered saline; Rb, retinoblastoma; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; T-G, Tris-Glycine.

***Address for reprints:** Department of Molecular, Cellular and Craniofacial Biology, ULSD, University of Louisville, Birth Defects Center, 501 S. Preston Street, Louisville, KY 40292, USA. FAX: 502-852-8309. e-mail: pisano@louisville.edu

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acts to tether the Rb family members to the promoter regions of genes, where the tethered Rb functions to either interfere with interactions between E2F and the transcriptional machinery (Luca *et al.*, 1998), or to recruit histone deacetylase and/or other co-repressors (Sellers *et al.*, 1995; Weintraub *et al.*, 1995; Brehm *et al.*, 1998; Ferreira *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998; Brehm and Kouzarides, 1999; Meloni *et al.*, 1999). The current model of E2F function postulates that withdrawal from active cycling is associated with accumulation of repressor complexes, particularly ones containing E2F-4 or -5 complexed to p130 and E2F-4 complexed to pRb (Helin, 1998; Nevins, 1998). Furthermore, differentiation also appears to be associated with up-regulation of E2F-4/p130 complexes, at least in myoblasts (Corbeil *et al.*, 1995; Kiess *et al.*, 1995; Wang *et al.*, 1995; Puri *et al.*, 1997).

In addition to complex formation, other factors regulate E2F function including: 1) synthesis and degradation, 2) subcellular redistribution and 3) post-translational modifications such as phosphorylation. The E2Fs are phosphorylated in a cell cycle-dependent fashion during the S and G_2 phases of the cell cycle resulting in both a reduction in their affinity for DNA (Dynlacht *et al.*, 1994; Xu *et al.*, 1994; Kitagawa *et al.*, 1995), and a predisposition to degradation (Vandel and Kouzarides, 1999). Phosphorylation also governs the interaction of E2F with the members of the Rb family. Although Rb phosphorylation reduces E2F/Rb interaction, phosphorylation of E2F can either increase or decrease complex formation depending on the site of phosphorylation (Fagan *et al.*, 1994; Peeper *et al.*, 1995; Yang and Sladek, 1997). Several of the E2Fs have been shown to be present in various phosphorylated forms which can be detected during immunoblot analysis as immunoreactive bands of higher molecular weight (Fagan *et al.*, 1994; Shin *et al.*, 1995; Yang and Sladek, 1997; Martelli and Livingston, 1999).

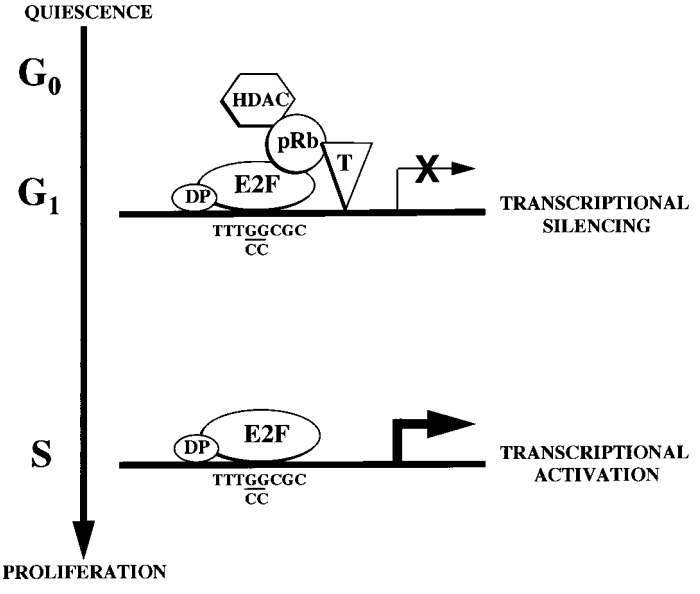

Fig. 1. Schematic representation of E2F-mediated effects on transcription. *E2F transcriptional "repressor" complexes are bound to the E2F consensus sequence in promoters during the G_0 and early G_1 phases of the cell cycle. Such repressor complexes consisting of E2F, its heterodimeric partner DP, and a hypophosphorylated member of the retinoblastoma family of tumor suppressor proteins (pRb), which can directly bind components of the transcriptional machinery (T) or can recruit histone-modifying enzymes such as histone deacytlase (HDAC), silence transcription of genes such as those necessary for G_1-S cell cycle progression and DNA synthesis. E2F transcriptional "activator" complexes, however, are bound to the E2F consensus sequence during late G_1 and S phases of the cell cycle, and when bound as such activate the varied genes necessary for G_1-S cell cycle progression and DNA synthesis.*

Most of the above characteristics of the E2Fs and their complexes have been delineated in neoplastic and overexpressing cell lines, and much less attention has been directed toward the E2Fs, their expression, and their role in control of the cell cycle during normal embryogenesis, a period in which tightly regulated spatio-temporal patterns of growth and differentiation are requisite for proper development. *In situ* hybridization has been used to examine the expression of E2Fs in the developing nervous system where high levels of E2F-1, -2 and -5 were found during proliferation of neuronal precursor cells while differentiation was accompanied by subsequent reductions in all three of these message levels (Dagnino *et al.*, 1997a). In contrast to neuronal tissue, epithelial proliferation was characterized by the expression of E2F-2 and -4 mRNAs, and differentiation was correlated with the appearance of E2F-5 and the down-regulation of E2F-2 and -4. The levels of E2F-1 and -3 transcripts in embryonic epithelium were found to be low and to remain constant during development. E2F-2 and -4 were the only members of the E2F family to be expressed in the subepithelial mesenchyme and only at low levels (Dagnino *et al.*, 1997b). Thus, it would appear that during embryogenesis, the levels of E2F mRNA are tissue-specific and change as a function of development.

Since embryonic cells/tissues are distinct from neoplastic cells and adult cells/tissues, and since little is known regarding the expression and/or function(s) of the E2Fs during development, we have examined the protein levels of the six currently identified E2Fs during murine embryonic and fetal development. In addition, comparisons have been made between select fetal and adult tissues with regard to the profile of E2F-1 through -6 expression. Moreover, since the mammalian secondary palate as a developing organ system constitutes a paradigm wherein development is contingent on tightly regulated spatio-temporal patterns of cell proliferation, apoptosis and cell transdifferentiation, all of which are achieved through regulation of cell cycle progression, we also have examined the expression of the E2F transcription factor family in the developing murine secondary palate during the crucial stages of palatogenesis (days 12 through 14 of gestation).

Results

E2F-1

Protein levels of E2F-1 were examined by western immunoblotting utilizing several different antibodies: KH-129 and KH-95 (mouse monoclonal antibodies), and C-20 (rabbit polyclonal antibody). The E2F-1 monoclonal antibody (KH-129) recognized at least 4 bands with apparent molecular weights of 63, 59, 57, and 55 kDa. These four immunoreactive E2F-1 forms were evident in palatal tissue from GD 12, 13 and 14 embryos. No substantial alteration in the pattern of expression of these four forms was observed during these three critical days of development of the secondary palate (Fig. 2A). When the pattern of expression in whole embryos from GD 9 to GD 18 was examined, or when individual adult and embryonic organs were compared, marked

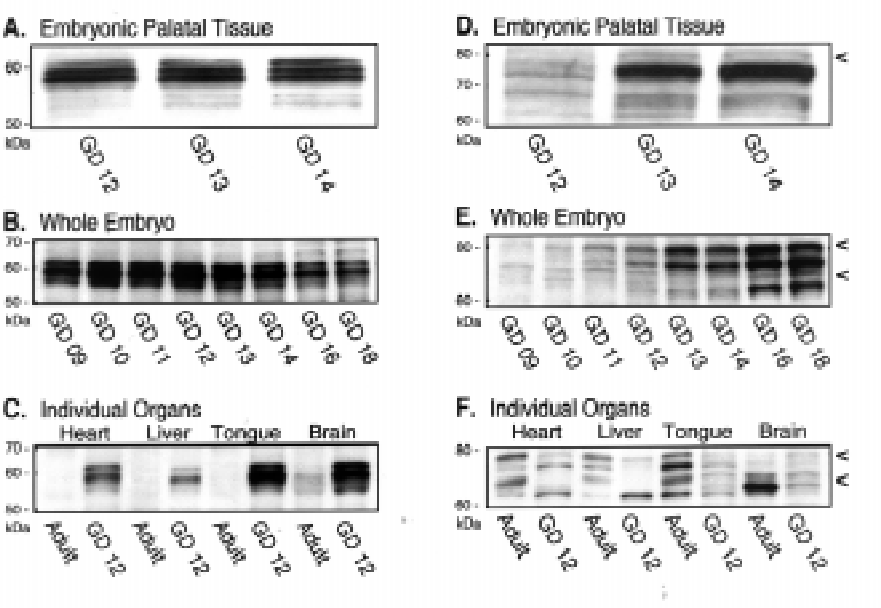

Fig. 2. Immunoblot analysis of E2F-1 expression. *Immunoblots were probed with either E2F-1 KH-129 mouse monoclonal* **(A,B and C)** *or KH-95 mouse monoclonal* **(D,E and F)** *antibodies. Molecular weights of the protein markers are indicated on the left of the panels; apparent molecular weights of the various E2F-1 protein bands are detailed in the text. Arrowheads to the right of the panels designate nonspecific immunoreactive bands, which were determined by probing the blot with only the secondary antibody, prior to detection with the primary and secondary antibodies.* **(A)** *Embryonic palatal tissue lysates probed with KH-129 antibody: lysates (60 μg of protein) prepared from murine embryonic palatal shelves dissected from embryos of 12, 13 and 14 days of gestation were separated by SDS-PAGE via 8-16% Tris-glycine (T-G) gels, transferred to PVDF membranes, probed and immunoreactive species detected by chemiluminescence.* **(B)** *Whole embryo lysates probed with KH-129 antibody: lysates (30 μg of protein) prepared from gestational day (GD) 9 through 18 murine embryos were separated via an 8% T-G gel.* **(C)** *Adult and embryonic organ lysates probed with KH-129 antibody: 25 μg of lysate protein prepared from heart, liver, tongue and brain of adult mice and GD 12 embryos were separated via an 8% T-G gel.* **(D)** *Embryonic palatal tissue lysates probed with KH-95 antibody: 90 μg of protein were separated via an 8% T-G gel.* **(E)** *Whole embryo lysates probed with KH-95 antibody: 30 μg of protein were separated via an 8% T-G gel.* **(F)** *Adult and embryonic organ lysates probed with KH-95 antibody: 25 μg of protein were separated via an 8% T-G gel.*

changes in the expression profile were seen. E2F-1 KH-129-immunoreactive protein levels in whole embryos consistently declined after GD 10 with the most prominent reductions occurring on GD 16 and GD 18 (Fig. 2B). Lysates from the brain, tongue, heart and liver of GD 12 embryos consistently demonstrated three or four KH-129 immunoreactive forms. The corresponding adult tissues exhibited little or no expression. The rank order of KH-129-immunoreactive protein levels in GD 12 embryonic tissues was tongue = brain > heart > liver (Fig. 2C).

The E2F-1 monoclonal antibody (KH-95) recognized several bands with apparent molecular weights between 63 and 78 kDa. In general, a doublet at 75-78 kDa was prominent in palatal tissue and sharply increased on day 13 of gestation with a further increase on GD 14 (Fig. 2D). A 68 kDa protein was observable on GD 12, which became a doublet with the appearance of a 65 kDa protein on GD 13. Expression of this doublet did not change substantially on GD 14. When whole embryo extracts were examined, both the 75-78 kDa and the 65-68 kDa complexes displayed up-regulation during development with significant increases on the gestational day 12 and 13 and thereafter (Fig. 2E).

Examination of adult and embryonic tissues revealed no universal pattern of expression of the KH-95-reactive proteins (Fig. 2F). Adult brain exhibited high levels of an immunoreactive protein with an apparent molecular weight of 70 kDa, which was only faintly detectable on GD 12. Two KH-95-reactive forms with apparent molecular weights of 66 and 77 kDa were found in adult tongue, while the tongue from GD 12 embryos manifested four very weakly expressed forms with apparent molecular weights of 66, 70, 74 and 77 kDa. Thus, in brain and tongue, the KH-95-reactive forms of E2F-1 were predominately found in adult tissue. By comparison, embryonic liver expressed a single form with an apparent molecular weight of 66 kDa. This form was dramatically down-regulated in adult liver, which also expressed a 74 kDa form. Embryonic heart weakly expressed both the 66 and 74 kDa forms and both were down-regulated in the adult tissue.

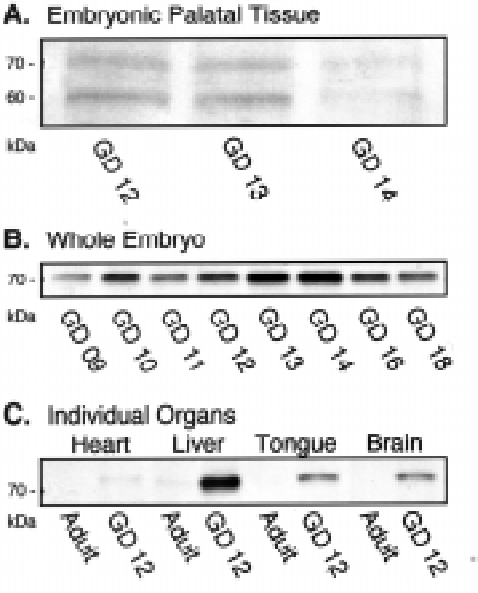

Fig. 3. Immunoblot analysis of E2F-2 expression. *Immunoblots were probed with the E2F-2 mouse monoclonal antibody TFE-25. Molecular weights of protein markers are indicated on the left of the panels; the apparent molecular weights of the various E2F-2 protein bands are detailed in the text.* **(A)** *Embryonic palatal tissue lysates (90 μg of protein) prepared from murine embryonic palatal shelves dissected from embryos of 12, 13 and 14 days of gestation were separated by SDS-PAGE electrophoresis on 8% T-G gels, transferred to PVDF membrane, probed and immunoreactive species detected by chemiluminescence.* **(B)** *Whole embryo lysates: protein lysates (30 μg of protein) from gestational day (GD) 9 through 18 murine embryos were separated on 8% T-G gels.* **(C)** *Adult and embryonic organ lysates: protein lysates (25 μg of protein) from heart, liver, tongue and brain of adult and GD 12 embryos were separated via 8% T-G gels.*

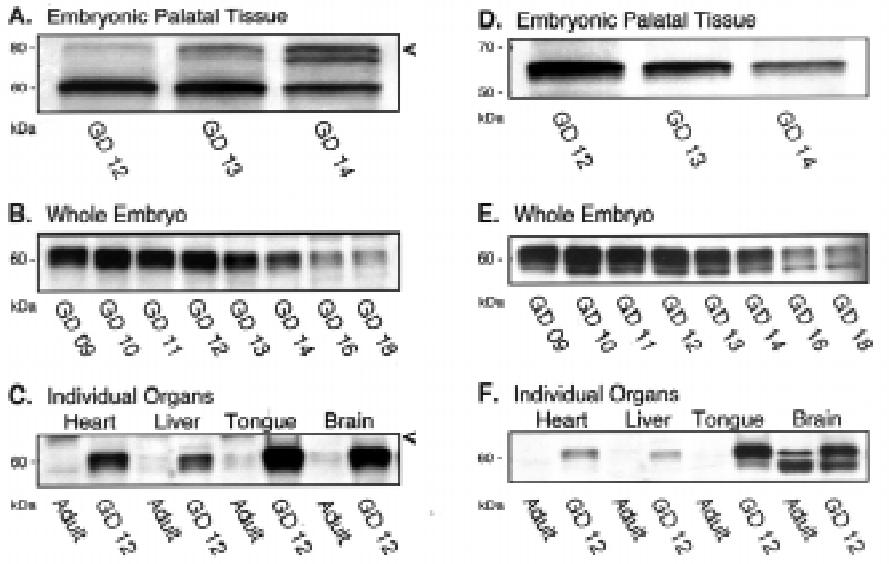

Fig. 4. Immunoblot analysis of E2F-3 expression. *Immunoblots were probed with either E2F-3 PG-37 mouse monoclonal* **(A,B and C)** *or N-20 rabbit polyclonal* **(D,E and F)** *antibodies. Molecular weights of the protein markers are indicated on left of the panels; the apparent molecular weights of the various E2F-3 protein bands are detailed in the text. Arrowheads to the right of the panels designate nonspecific immunoreactive bands, which were determined by probing the blot with only the secondary antibody, prior to detection with the primary and secondary antibodies.* **(A)** *Embryonic palatal tissue lysates probed with PG-37 antibody: lysates (60 μg of protein) prepared from murine embryonic palatal shelves dissected from embryos of 12,13 and 14 days of gestation were separated by SDS-PAGE on 8-16% T-G gels, transferred to PVDF membrane, probed, and immunoreactive species detected by chemiluminescence.* **(B)** *Whole embryo lysates probed with PG-37 antibody: lysates (30 μg of protein) prepared from gestational day (GD) 9 through 18 murine embryos were separated via 8% T-G gels.* **(C)** *Adult and embryonic organ lysates probed with PG-37 antibody: lysates (25 μg of protein) prepared from heart, liver, tongue and brain of adult and GD 12 embryos were separated via 8% T-G gels.* **(D)** *Embryonic palatal tissue lysates probed with N-20 antibody: lysates (60 μg of protein) were separated via 8% T-G gels.* **(E)** *Whole embryo lysates probed with N-20 antibody: lysates (30 μg of protein) were separated via 8% T-G gels.* **(F)** *Adult and embryonic organ lysates probed with N-20 antibody: lysates (25 μg of protein) were separated via 8 % gels. Panels C and F are sequential probings of the same immunoblot with the two E2F-3 antibodies. Note the striking appearance of immunoreactive proteins in samples of adult brain when probed with the E2F-3 N-20 antibody as compared to the PG-37 antibody.*

The E2F-1 rabbit polyclonal antibody (C-20) recognized the same forms and revealed a similar developmental pattern of E2F-1 as the KH-129 monoclonal antibody. It was also weakly reactive with the 75-78 kDa E2F-1 form recognized by KH-95 monoclonal antibody (data not shown).

E2F-2

Two antibodies were used to examine the expression of E2F-2: TFE-25, a mouse monoclonal and C-20, a rabbit polyclonal antibody which although reacting nonspecifically with a number of proteins, also recognized those proteins immunoreactive with the TFE-25 monoclonal antibody (data not shown). Thus, the E2F-2 polyclonal antibody (C-20) was used only to confirm the results observed with the E2F-2 monoclonal antibody (TFE-25).

Extremely low levels of two immunoreactive forms of E2F-2 were detected in lysates of embryonic palatal tissue with apparent molecular weights of 62 and 72 kDa. Both species were down-regulated on GD 14 (Fig. 3A). Only the 72 kDa immunoreactive form of E2F-2 was observed in whole embryos, and it followed a developmental pattern unique from that of the other E2F family members. Consistently low levels of expression were seen on GD 9. Levels fluctuated between GD 10 and 13, peaked on GD 14 and progressively fell thereafter (Fig. 3B). In individual organs, the E2F-2 monoclonal antibody (TFE-25) recognized two slower migrating species at 72 kDa and 74 kDa. Their expression was limited almost exclusively to embryonic organs (Fig. 3C). Adult liver yielded a signal on immunoblots but only after extreme overexposure. Comparing E2F-2 levels in embryonic organs, the content of liver far exceeded that of brain or tongue. However, the relative distribution of the two species was tissue-specific with the liver expressing mainly the 72 kDa isoform and the brain and tongue preferentially expressing the 74 kDa isoform.

E2F-3

The N-20 rabbit polyclonal and the PG-37 mouse monoclonal antibodies were used to determine the expression of E2F-3. In lysates of developing palatal tissue, both antibodies reacted strongly with proteins with apparent molecular weights of 62 and 59 kDa (Fig. 4A and D). The monoclonal antibody, PG-37 also recognized proteins of 77, 68, and 49 kDa; however these bands on immunoblots were considerably weaker than the major doublet. The N-20 polyclonal antibody also reacted with these secondary forms but less strongly than the PG-37 monoclonal antibody. The levels of the 62-59 kDa E2F-3 complex in palatal tissue declined on GD 14 while the expression of the PG-37-reactive 77 kDa form was up-regulated.

Whole embryos exhibited a decline in expression of the 62-59 kDa E2F-3 complex from GD 10 through GD 18 (Fig. 4B and E). The E2F-3 polyclonal antibody (N-20) also reacted with a 57 kDa protein in whole embryo lysates which was not apparent in lysates from palatal tissue but which followed the pattern of decline with development seen for the 62-59 kDa complex (Fig. 4E).

The preferential expression of E2F-3 species in embryonic tissues was also observed in heart, liver and tongue. When the same blot was sequentially probed with the two antibodies, the PG-37 immunoreactive forms of E2F-3 exhibited a strong preferential expression in all embryonic tissues including brain, with the rank order of expression in GD 12 tissues being tongue > brain > heart > liver (Fig. 4C). When the N-20 antibody was used, E2F-3 immunoreactive forms were preferentially expressed in all embryonic tissues except brain (Fig. 4F). In brain, the E2F-3 polyclonal antibody (N-20) recognized proteins with apparent molecular weights of 60 kDa and 57 kDa which were not detected by the E2F-3 monoclonal antibody (PG-37). The 57 kDa species recognized by N-20 appeared to be similar to the N-20-reactive species present

in whole embryo, and was expressed in both embryonic day 12 and adult brain, with adult brain exhibiting higher levels than the embryonic organ. The 60 kDa species was not seen in any other adult tissues; although, it was not possible to ascertain whether it was a component of the 62-59 kDa embryonic complex or a separate species. In either event, adult brain differs significantly from the other adult tissues examined in this study, in that it contains high levels of E2F-3 immunoreactive proteins which are only recognized by the N-20 polyclonal antibody to E2F-3.

E2F-4

Two antibodies were used to examine the levels of E2F-4 protein: TFE-42, a mouse monoclonal antibody and C-20, a rabbit polyclonal antibody. Both antibodies appeared to recognize the same proteins, the most highly expressed of which had apparent molecular weights of 66, 62, and 59 kDa. However, two lesser bands with apparent molecular weights of 73 and 77 kDa were consistently observed with both antibodies. Since both antibodies recognized the same E2F-4 species, only those results obtained with the C-20 antibody are presented.

In the embryonic palate, levels of the three major forms of E2F-4 decreased as a function of development, with the most marked reduction on day 14 of gestation (Fig. 5A). Levels in whole embryos were maximal on GD 9 or 10, and gradually decreased as development proceeded (Fig. 5B). A strong embryonic preference in expression was also observed between adult and embryonic tissues (Fig. 5C). The rank order of E2F-4 expression in embryonic tissues was liver > tongue = brain > heart. Although levels were substantially lower in the adult tissues, brain exhibited the highest level of E2F-4 expression among the adult organs.

E2F-5

Protein levels of E2F-5 were examined using MH-5, a mouse monoclonal antibody, and E-19, a rabbit polyclonal antibody, each antibody recognizing separate immunoreactive forms. The MH-5 monoclonal antibody reacted with two proteins with apparent molecular weights of 59 kDa and 42 kDa. No appreciable difference in the expression of these two species could be observed in embryonic palatal tissue on GD 12, 13 and 14 (Fig. 6A). However, differential expression was observed during development in whole embryos with the 59 kDa form increasing and the 42 kDa form decreasing with gestational age from days 9 through 18 of development (Fig. 6B). Thus, on GD 9 the faster migrating species predominated but by GD 16 the slower migrating species was more prominent.

This pattern was dramatically illustrated when comparing adult and embryonic tissues (Fig. 6C). The 59 kDa species of E2F-5 was highly expressed in adult organs where it was the only detectable form, and exhibiting a rank order of heart = liver ≥ tongue > brain. The corresponding tissues from GD 12 embryos exhibited considerably lower levels of the 59 kDa immunoreactive form of E2F-5 but also displayed the typically embryonic 42 kDa species.

The second E2F-5 antibody, E-19 rabbit polyclonal, recognized a single protein with an apparent molecular weight of 68 kDa, which appeared to be a predominately embryonic form of E2F-5. The E-19-reactive species was most highly expressed in palatal tissue on GD 12 and 13 and was down-regulated on GD 14 (Fig. 6D). Expression in whole embryo diminished from GD 10 through GD 18 (Fig. 6E). The embryonic nature of this species was most dramatically illustrated in studies in which the levels of E-19-reactive form of E2F-5 were examined in adult and embryonic organs (Fig. 6F). Embryonic tissues all displayed a prominent expression of the 68 kDa species of E2F-5 with their rank order being brain = liver >heart > tongue. However, this form of E2F-5 was hardly detectable in the same tissues from adult animals.

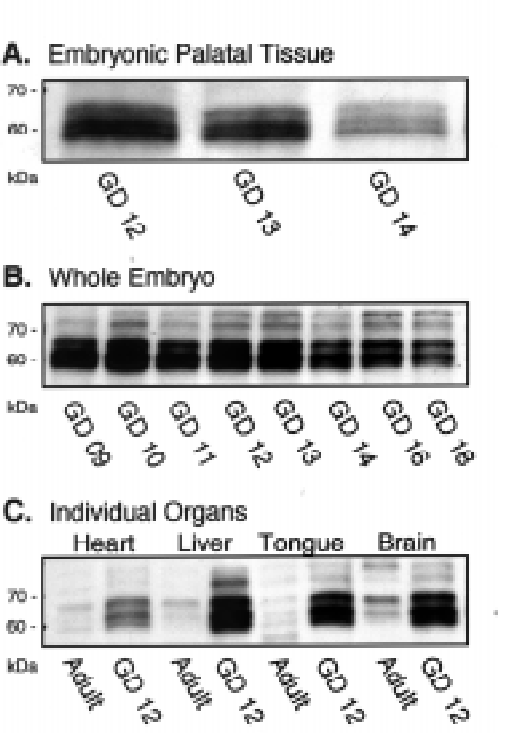

Fig. 5. Immunoblot analysis of E2F-4 expression. *Immunoblots were probed with the E2F-4 C-20 rabbit polyclonal antibody. Molecular weights of the protein markers are indicated on the left of the panels; the apparent molecular weights of the various E2F-4 protein bands are detailed in the text. The same and similar immunoblots were also probed with the E2F-4 TFE-42 mouse monoclonal antibody with virtually identical results.* **(A)** *Embryonic palatal tissue lysates: lysates (90 μg of protein) prepared from murine embryonic palatal shelves dissected from embryos of 12,13 and 14 days of gestation were separated by SDS-PAGE on 8% T-G gels, transferred to PVDF membrane, probed, and immunoreactive species detected by chemiluminescence.* **(B)** *Whole embryo lysates: tissue lysates (30 μg of protein) from gestational day (GD) 9 through 18 murine embryos were separated via 8% T-G gels.* **(C)** *Adult and embryonic organ lysates: lysates (25 μg of protein) from heart, liver, tongue and brain of adult and GD 12 embryos were separated via 8% T-G gels.*

E2F-6

Only one commercially available E2F-6 antibody appeared to recognize a specific protein on immunoblots of *murine* lysates. The N-19 goat polyclonal antibody reacted with a protein with an apparent molecular weight of 28 kDa. Detectable levels of the N-19 immunoreactive protein were observable in the developing palate, being highest on GD 12 and successively down-regulated on days 13 and 14 of gestation (Fig. 7A). This immunoreactive species was most highly expressed during early embryogenesis (GD 9 and 10) and was progressively down-regulated as gestation proceeded from day 10 through day 18 (Fig. 7B). The E2F-6 N-19 immunoreactive form was preferentially expressed in embryonic

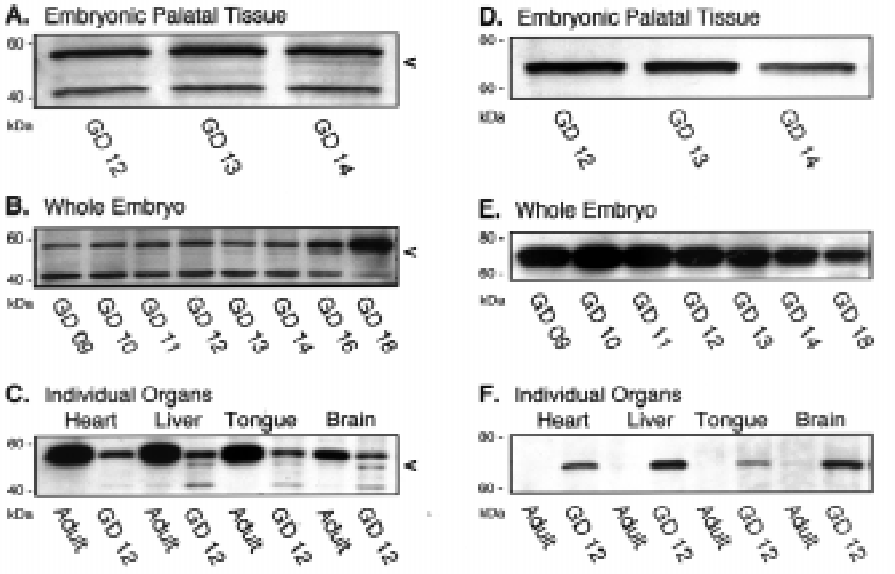

Fig. 6. Immunoblot analysis of E2F-5 expression. *Immunoblots were probed with either E2F-5 MH-5 mouse monoclonal* **(A,B and C)** *or E-19 rabbit polyclonal* **(D,E and F)** *antibodies. Molecular weights of the protein markers are indicated on the left of the panels; the apparent molecular weights of the various E2F-5 protein bands are detailed in the text. Arrowheads to the right of the panels designate nonspecific immunoreactive bands, which were determined by probing the blot with only the secondary antibody, prior to detection with the primary and secondary antibodies.* **(A)** *Embryonic palatal tissue lysates probed with MH-5 antibody: lysates (60 μg of protein) prepared from murine embryonic palatal shelves dissected from embryos of 12,13 and 14 days of gestation were separated by SDS-PAGE on 8-16% T-G gels, transferred to PVDF membranes, probed, and immunoreactive species detected by chemiluminescence.* **(B)** *Whole embryo lysates probed with MH-5 antibody: tissue lysates (33 μg of protein) from gestational day (GD) 9 through 18 murine embryos were separated via 8-16% T-G gels.* **(C)** *Adult and embryonic organ lysates probed with MH-5 antibody: lysates (20 μg of protein) from heart, liver, tongue and brain of adult and GD 12 embryos were separated via 8-16% T-G gels.* **(D)** *Embryonic palatal tissue lysates probed with E-19 antibody: lysates (60 μg of protein) prepared from murine embryonic palatal shelves dissected from embryos of 12,13 and 14 days of gestation were separated by SDS-PAGE on 8-16% T-G gels, transferred to PVDF membranes, probed, and immunoreactive species detected by chemiluminescence.* **(E)** *Whole embryo lysates probed with E-19 antibody: tissue lysates (25 μg of protein) from GD 9 through 18 murine embryos were separated via 8-16% T-G gels.* **(F)** *Adult and embryonic organ lysates probed with E-19 antibody: lysates (25 μg of protein) from heart, liver, tongue and brain of adult and GD 12 embryos were separated via 8-16% T-G gels.*

organs, with liver, brain and tongue exhibiting the highest levels and heart the lowest levels of expression (Fig. 7C). The only adult organs to express E2F-6 were liver and brain, although the levels in brain were extremely low.

Discussion

Because the craniofacial region is one of the most rapidly growing and developing areas in the embryo, it is highly susceptible to malformations. Craniofacial anomalies occur with a frequency of 1 in 600 live births annually in the United States with 65 percent of these affected newborns manifesting clefts of the lip and/or palate (Niermeyer and Van Der Meulen, 1990). A singular commonality in cases of orofacial clefting, in humans and animal models, is a notable growth insufficiency of the lip, palate, and/or surrounding tissues. In fact, numerous cleft-producing teratogens, such as retinoic acid, alcohol, glucocorticoids, phenytoin, and heavy metals, to name a few, have been shown to be marked inhibitors of cellular proliferation (Kochhar, 1968; Nanda and Romeo, 1978; Salomon and Pratt, 1978; Olson and Massaro, 1980; Tassinari *et al.*, 1981; Weston *et al.*, 1994). Although the mechanism of action of each of these teratogenic agents is quite disparate, each is capable of perturbing orofacial growth via total inhibition or partial down-regulation of cellular proliferation, substantiating the criticality of proper spatio-temporal patterns of proliferation to normal palatogenesis. The developing secondary palate has proven to be a valuable model system, not only for gaining insight into the etiology of palatal clefts, but more importantly for developing insight into the mechanisms by which growth factors and morphogens orchestrate patterns of cellular proliferation and differentiation, thereby giving rise to the genesis of tissues and organs, and overall body form (Greene, 1989; Greene and Pisano, 1989; Greene *et al.*, 1991, 1998). Certain events critical for palatal ontogenesis, such as spatio-temporally regulated patterns of cellular growth, differentiation and apoptosis, are under the immediate control of the cell cycle, and hence an understanding of the means by which the cell cycle orchestrates these processes during embryonic palate development is crucial for understanding the intricacies of palate development.

Within the past decade, members of the E2F family of transcription factors have been found to be key effectors of the cell cycle (Martin et al., 1995; Bernards, 1997). Their role in cellular proliferation, cell growth arrest and apoptosis has been well established (Nevins, 1998; DeGregori *et al.*, 1997). The E2F transcription factors exhibit dual transcriptional functions depending on whether they are present as “free E2F” (i.e. E2F dimerized to its transcription factor partner DP) or “complexed E2F” (i.e. E2F bound to the retinoblastoma tumor suppressor proteins pRb, p107, p130). When present as “free E2F”, E2F is a transcriptional activator, stimulating the expression of key components of the cell cycle machinery (e.g. cyclins A and E, cdc25A, E2Fs, pRb, p107 and various enzymes necessary for S-phase DNA synthesis). When present as “complexed E2F”, E2F is a transcriptional repressor, actively silencing the expression of many of these same genes. Thus, the E2F transcription factors are pivotal in the balance between stimulation and inhibition of cellular proliferation, and are exquisitely poised to provide the precise spatial and temporal control of growth required for proper morphogenesis. However, little is known regarding the expression and/or role of the E2Fs in embryogenesis. In the present study, we report on the expression of the E2F family of transcription factors in murine development with particular emphasis on its expression in the murine embryonic palate during the critical days of palate development (GD 12-14).

Analysis of embryos on various gestational days and select embryonic tissues indicates that each of the currently identified E2F proteins is present at detectable levels during murine embryo-

genesis and that each appears to exhibit tissue- and temporal-specific patterns of expression (see Table 1 for overview of results). Although most E2F species are down-regulated as gestation proceeds, the differential patterns of expression suggest that precise combinations of E2Fs may be required for the varied processes occurring during organogenesis and later fetal growth. The only E2Fs to be up-regulated as a function of gestational development are the E2F-1 isoforms recognized by the KH-95 antibody and the E2F-5 isoform recognized by the MH-5 antibody. Complexes of E2F-5 (and also E2F-4) with the retinoblastoma protein p130 have been found to accumulate in varied cell types during quiescence and differentiation (Cobrinik *et al.*, 1993; Ginsberg *et al.*, 1994; Mayol *et al.*, 1995; Sardet *et al.*, 1995; Wolf *et al.*, 1995; Sears *et al.*, 1997). Thus, the marked up-regulation of E2F-5 during the later stages of gestation would be consistent with a role for E2F-5 in the maintenance of differentiated phenotypes in the fetus. Reprobing of the whole embryo immunoblots used in the present investigation, revealed a simultaneous up-regulation of total p130 protein on GD 16 and 18, and a marked reduction in phosphorylated form of p130 on GD 18 (unpublished observations). Such expression patterns of p130 and E2F-5 are consistent with formation of E2F-5/p130 complexes which would act to stabilize the differentiated cellular phenotypes of the developing fetuses.

The other E2F family member implicated in quiescence, E2F-4, (Ginsberg *et al.*, 1994; Sardet *et al.*, 1995; Sears *et al.*, 1997), was down-regulated in whole embryos as embryogenesis proceeded. E2F-4 may be more versatile than the other E2Fs since it has been shown to form complexes with all three members of the Rb family of proteins and to be expressed during the entire cell cycle (Moberg *et al.*, 1996). Furthermore, overexpression of E2F-4, but not E2F 5, can selectively activate the genes of some of the cell cycle components containing E2F-binding sites (DeGregori *et al.*, 1997). Thus, although E2F-4 and E2F-5 share substantial sequence homology (Dyson, 1998), they may play distinct roles during embryogenesis. E2F-2 and E2F-3 also share a high degree of homology, but display differing abilities to activate cell cycle-regulated genes (DeGregori, *et al.*, 1997). Although expression of both E2F-2 and E2F-3 was down-regulated in whole embryos as gestation proceeded, the two proteins displayed different temporal patterns of down-regulation which may reflect differences in the relative importance of the various components of the cell cycle apparatus at different stages of development.

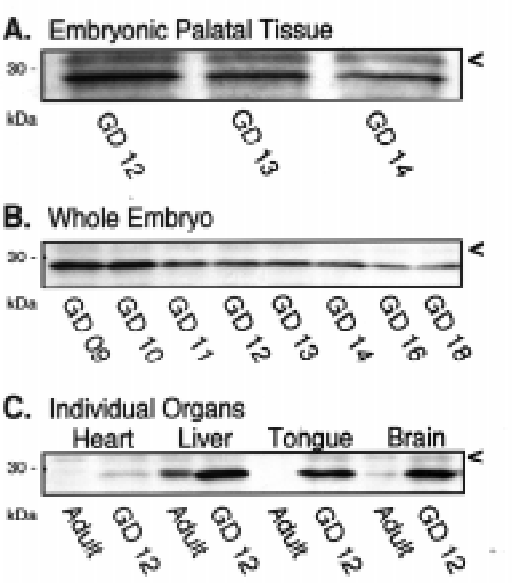

Fig. 7. Immunoblot analysis of E2F-6 expression. *Immunoblots were probed with E2F-6 N-19 goat polyclonal antibody. Molecular weights of the protein markers are indicated on the left of the panels; the apparent molecular weights of the various E2F-6 protein bands are detailed in the text. Arrowheads to the right of the panels designate non-specific immunoreactive bands, which were determined by probing the blot with only the secondary antibody, prior to detection with the primary and secondary antibodies.* **(A)** *Embryonic palatal tissue lysates: lysates (90 μg of protein) prepared from murine embryonic palatal shelves dissected from embryos of 12,13 and 14 days of gestation were separated by SDS-PAGE on 8-16% T-G gels, transferred to PVDF membrane, probed, and immunoreactive species detected by chemiluminescence.* **(B)** *Whole embryo lysates: tissue lysates (40 μg of protein) from gestational day (GD) 9 through 18 murine embryos were separated via 8-16% T-G gels.* **(C)** *Adult and embryonic organ lysates: lysates (40 μg of protein) from heart, liver, tongue and brain of adult and GD 12 embryos were separated via 8-16% T-G gels.*

E2F-6 shares only limited homology with the other members of the E2F family. It contains DNA binding and dimerization domains but lacks both the transactivation domain and the retinoblastoma

TABLE 1

E2F EXPRESSION PROFILES DURING MURINE DEVELOPMENT

E2F[1]	EXPRESSION IN DEVELOPING PALATE[2]	EXPRESSION DURING MURINE DEVELOPMENT DAYS 8 -18 OF GESTATION	PREFERENTIAL EXPRESSION IN ADULT OR DEVELOPING TISSUE[3]
E2F-1 (KH-129)	Constant	Peaks at GD 11-12	Developing
E2F-1 (KH-95)	Up-regulated	Progressively up-regulated from GD 9-18	Adult (Brain and Tongue) Developing (Heart and Liver)
E2F-2 (TFE-25)	Low levels; down-regulated	Peaks at GD 13-14	Developing
E2F-3 (PG-37)	Marginally down-regulated	High levels GD 9-12, followed by progressive down-regulation	Developing
E2F-3 (N-20)	Down-regulated	High levels GD 9-12, followed by progressive down-regulation	Developing[4]
E2F -4 (C-20)	Down-regulated	High levels GD 9-13, followed by progressive down-regulation	Developing
E2F-4 (TFE-42)	Down-regulated	High levels GD 9-13, followed by progressive down-regulation	Developing
E2F-5 (MH-5)	Constant	42 kDa: progressively down-regulated 57 kDa: markedly up-regulated GD 18	Developing Adult
E2F-5 (E-19)	Marginally down-regulated	Peaks at GD 10, followed by progressive down-regulation	Developing
E2F-6 (N-19)	Down-regulated	Peaks at GD 9-10; followed by progressive down-regulation	Developing

1 - E2F family members are listed in column 1 by primary antibody utilized in the immunodetection analyses.
2 - Developing palatal tissue examined during the critical period of murine palatogenesis: GD 12 through GD 14.
3 – Indication of whether particular antibody used preferentially detects expression in adult or developing tissues.
4 - Also highly expressed in adult brain (see text).

binding domain found in the carboxy terminus of other E2Fs (Morkel *et al.*, 1997; Cartwright *et al.*, 1998; Gaubatz *et al.*, 1998; Trimarchi *et al.*, 1998). The ability of E2F-6 to occupy E2F consensus sequences within promoters, without transactivating gene expression, suggests that E2F-6 can repress transcription. Expression of E2F-6 in murine development was highest earlier in gestation (days 9 and 10) and lowest shortly before birth. This pattern is the converse of that for components of repressor complexes, E2F-5 and p130, which may reflect the reliance of the embryo on different mechanisms of repression of E2F-regulated genes at different stages of embryogenesis.

Undoubtedly, the most complex pattern of developmental expression was exhibited by E2F-1. It exhibited the greatest number of isoforms, none of which were universally recognized by any single E2F-1 antibody. All three E2F-1 antibodies used in the present study recognized different proteins with different developmental and tissue profiles. The KH-95 antibody, with an epitope mapping to the Rb-binding domain (aa 342-386 of the human protein), recognizes at least five bands, two immunoreactive doublets (75-78 and 65-68 kDa) and a single band with an apparent molecular weight of 63 kDa, all of which are up-regulated as a function of development in the whole embryo. The KH-129 antibody with an adjacent epitope (aa 386-409) reacts with at least four different species with apparent molecular weights of 63, 59, 57 and 55 kDa, which are down-regulated as gestation proceeds. The C-20 polyclonal antibody whose epitope encompasses the entire carboxy terminus tends to follow the pattern of KH-129, although it is very weakly reactive with the 75-78 kDa doublet recognized by KH-95. The fact that there is some cross-reactivity (albeit weak) between the three antibodies tends to confirm the veracity of our results. The multiplicity of immunoreactive E2F-1 species has also been reported in murine fibroblast cell lines overexpressing E2F-1 (Yang and Sladek, 1997). Interestingly, these investigators found that the slower migrating, more highly phosphorylated forms of E2F-1 were preferentially bound to pRb. In the present study, the slower migrating species of E2F-1 were preferentially recognized by the antibody raised against the Rb-binding site, suggesting that hyperphosphorylation may be a general mechanism whereby the affinity or availability of the Rb-binding domain is enhanced.

E2F-1 is efficiently phosphorylated by cyclin A-dependent kinases resulting in down-regulation of its DNA-binding affinity (Dynlacht *et al.*, 1994; Krek *et al.*, 1994; Xu *et al.*, 1994; Kitagawa *et al.*, 1995; Krek and Livingston, 1995). The consequent decrease in the transactivating potential of the phosphorylated form of E2F-1 is one of the postulated mechanisms for terminating E2F activity during late S phase (Dyson, 1998). Thus, a shift to more phosphorylated forms of E2F-1 in whole embryos as seen in the present study could be congruent with a slowing of proliferation as organogenesis concludes and gestation proceeds. The effect of phosphorylation on the properties of the other E2Fs has received less attention; however, differentially phosphorylated forms of E2F-4 and -5 have been reported (Ginsberg *et al.*, 1994; Vaishnav *et al.*, 1998), and correlated to changes in rates of cell proliferation. Quiescence of primary hematopoietic cells has been associated with a shift to hyperphosphorylated E2F-4 (Thomas *et al.*, 1998). In the current investigation, multiple species of E2F-1, -3, -4 and -5 were observed in individual tissues as well as in whole mouse embryo extracts. Preliminary data from our laboratory suggest that these species are due to differential phosphorylation of these E2F isoforms (manuscript in preparation). Although it is premature to speculate about the role of individual phosphoforms, the finding that E2F-1 (KH-95)-reactive species exhibit an expression pattern which is diametrical to other E2F-1 species and to other E2Fs suggests that phosphorylation may be an important regulatory mechanism governing the function of E2F during embryogenesis.

Comparisons of E2F-1 expression in adult versus embryonic tissues confirmed the reduction of E2F-1 (KH-129)-reactive forms with development and maturation. All of the adult tissues examined in this study exhibited lower levels of the species recognized by KH-129 than did their embryonic counterparts. In contrast, the pattern of expression of the KH-95-reactive isoforms was not uniform. Expression in tongue and brain followed the pattern exhibited by whole embryos, wherein expression was up-regulated with development. The converse was observed for E2F-1 expression in adult liver and heart wherein the isoforms recognized by KH-95 were downregulated with development. These differences in expression cannot be related to terminal differentiation since both neurons and cardiac muscle are terminally differentiated cell types. Adult brain displayed not only high levels of E2F-1 (KH-95)-reactive proteins but also appreciable levels of proteins which were uniquely recognized by the E2F-3 (N-20) antibody. The N-20 and PG-37 antibodies recognized the same species in all other tissues examined (a species which predominated in embryonic but not adult organs). This raises several intriguing questions: 1) Why should a terminally differentiated tissue, which will never again visit S phase, express such high levels of E2Fs? 2) Why are these two particular E2Fs expressed in adult brain? 3) Why are they not similarly expressed in other terminally differentiated tissues such as cardiac muscle which exhibits high levels of the MH-5 immunoreactive form of E2F-5, an E2F typically found in other adult tissues? This invites the speculation that various E2Fs have other tissue-specific functions in terminally differentiated tissues.

One function of the E2F family which may be relevant in terminally differentiated tissues is the induction of apoptosis. Overexpression of E2F-1 not only drives quiescent cells into S phase but also induces apoptotic cell death (Johnson *et al.*, 1993; Dobrowolski *et al.*, 1994; Qin *et al.*, 1994; Kowalik *et al.*, 1995; Lukas *et al.*, 1996; DeGregori *et al.*, 1997). Recently the apoptotic potentials of E2F-2 and -3 have also been demonstrated (Vigo *et al.*, 1999). The ability of E2F to induce apoptosis appears to be associated with its capacity to repress transcription (Phillips *et al.*, 1997). Thus, induction of apoptosis following terminal cell differentiation would be consistent with the presence of hyperphosphorylated forms of E2F-1 (i.e. those recognized by the KH-95 antibody) in the adult brain which might preferentially bind to hypophosphorylated forms of the Rb family. Apoptosis within developing nervous tissue is a hallmark of $Rb^{-/-}$ mice (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992) and is partially overcome by the simultaneous knockout of E2F-1 gene expression (Yamasaki *et al.*, 1998). Thus, E2F/pRb complexes may function in the death of neurons and/or glial cells and a reduction of E2F/pRb complexes might be protective during brain insults.

Expression of the E2Fs in embryonic palatal tissue during the critical period of murine palate development generally followed patterns similar to those seen in whole embryos. E2F-1 (KH-129)- and E2F-5 (MH-5)-reactive forms were similar on GD 12, 13 and 14. E2F-2, E2F-3, E2F-4 and the E2F-5 (E-19)-reactive forms were down-regulated on GD 14, a time when growth rates slow to match

those of other cranial structures. Only the E2F-1 (KH-95)-reactive form was up-regulated. Such a species may play a role in later apoptotic or differentiative events within the developing palate, rather than in proliferation. The differential patterns of E2F expression during development of the palate suggest that varied E2Fs may have discrete roles in the differentiative, proliferative and apoptotic events occurring during palatogenesis. However, in order to begin to interpret the changing patterns of E2F expression in the developing palate, the exceedingly complex nature of embryonic palate formation and the striking heterogeneity of the tissue must be taken into account. The embryonic palate consists of neuroectodermally-derived mesenchyme cells surrounded by a multilayered epithelium with three discrete phenotypes [stratified squamous oral epithelium, pseudostratified columnar nasal epithelium, and a unique medial edge epithelium (MEE) capable of apoptosis and transdifferentiation into a mesenchymal phenotype]. At any one point in time, these varied cell types are concurrently undergoing cellular proliferation, differentiation and apoptosis, all of which are dependent upon precise control (both temporally and spatially) of entry, exit and progression through the cell cycle. Thus, the E2Fs, as regulators of the cell cycle, are likely factors involved in palatogenesis. However, without the availability of antibodies capable of localizing the various E2Fs and their isoforms to discreet cell populations, it would be premature to speculate on the exact role of the individual E2Fs in palate development. Data presented in the present study has served primarily to target individual E2Fs for further investigation with regard to their potential role in growth and differentiation of the palate.

The changing patterns of E2F protein levels in the embryo and developing palate noted in the present study offer fascinating and suggestive clues regarding their function during development. Since multiple mechanisms are involved in the control of E2F transcriptional activity, including: 1) E2F complex formation with its dimerization partners, DP1 and DP2, with the retinoblastoma phosphoproteins, and with the nuclear coactivator CBP; 2) modulation of E2F phosphorylation state; 3) subcellular redistribution and nuclear localization of E2F; and 4) E2F degradation via ubiquitination, further investigations of E2F function during development will require integration of results from analysis of the above mechanisms with changes in E2F protein levels.

Materials and Methods

Animals

Mature male and female ICR mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were housed in rooms with a 12 hour alternating light and dark cycle and were maintained on Purina mouse chow and water *ad libitum*. Timed pregnancies were obtained by overnight mating of a single mature male with two nulliparous females. The presence of a vaginal plug was considered to be evidence of mating and the time designated as 0 days of gestation (GD 0).

Tissue preparation

Pregnant and nonpregnant female mice were euthanized by carbon dioxide asphyxiation and the gravid uteri or other adult tissues removed and placed in phosphate-buffered saline (PBS) on ice. Embryonic and adult tissues were dissected, minced, and washed twice with Ca^{++}- and Mg^{++}-free PBS. Embryonic tissues were homogenized in ground glass combination conical/cylindrical tissue grinders at 4ºC in an appropriate volume of lysis buffer [250 mM NaCl, 50 mM Tris (pH 7.4), 5 mM EDTA, 0.1%(v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM NaF, 1 mM sodium orthovanadate, 10 μg/ml leupeptin and 50 μg/ml aprotinin]. Adult tissues were homogenized in the above buffer using a Tekmar Tissumizer™ at half maximal speed for 30 to 45 sec at 4ºC. All homogenates were sonicated for 30 sec at 4ºC and allowed to sit on ice for 30 min with vigorous vortexing every 5 min, then cleared by centrifugation for 10 min at 13,000x g at 4ºC. Total protein in the extracts was determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

Immunoblotting

Appropriate amounts of lysate (50-90 μg of protein per lane for 5 well gels and 20-40 μg of protein for 10 well gels) were denatured by boiling in 2X Laemmli sample buffer (Laemmli, 1970) and the proteins separated by SDS-PAGE electrophoresis at 125 volts using either 8% or 8-16% polyacrylamide Tris-glycine gels (Novex, San Diego, CA) followed by electrophoretic transfer (30 volts for 2 h) of the proteins to PVDF membranes. Gels were subsequently stained with Coomassie blue (Sasse and Gallagher, 1991) and membranes with 0.1% fast green to visualize proteins and ensure the efficiency of transfer. Blots were blocked by incubation in 5% non-fat dry milk in TBST buffer (50 mM Tris, pH 7.6; 150 mM NaCl; 0.1% Tween-20) for 1 h at room temperature. Antibodies were prepared by diluting in blocking solution and the blots incubated with primary antibody for 1-1.5 h at room temperature, washed extensively and incubated for 0.5-0.75 h at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody. Immune complexes were detected using the ECL-Plus™ chemiluminescent detection system (Amersham Pharmacia Biotech, Arlington, IL) according to the manufacturer's instructions. "No primary antibody" control immunodetection was performed on each blot prior to blotting with the primary antibody in order to distinguish between specific immunoreactive bands and nonspecific bands due to interaction of the secondary antibody with endogenous proteins. Immunoblots of palatal shelves and adult/embryonic organs were replicated with similar results on a minimum of three complete sets of tissue samples. Whole embryo immunoblots were repeated on two sets of embryos.

The following primary antibodies were used: E2F-1 (C-20), E2F-1 (KH-95), E2F-2 (C-20), E2F-3 (N-20), E2F-4 (C-20), E2F-5 (MH-5), E2F-5 (E-19), E2F-6 (N-19) from Santa Cruz Biotechnology (Santa Cruz, CA), and E2F-1 (KH-129), E2F-2 (TFE-25), E2F-3 (PG-37), and E2F-4 (TFE-42) from NeoMarkers (Union City, CA). Secondary antibodies included goat anti-rabbit IgG (Santa Cruz Biotechnology), rabbit anti-mouse IgG_1 and IgG_{2a} (Zymed Laboratories, San Francisco, CA).

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