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Expression of cellular oncogenes during embryonic and fetal development of the mouse

(embryogenesis)

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ABSTRACT **Cellular oncogenes are conserved with great fidelity across a broad span of evolution. This avid conservation suggests possible roles in critical physiologic functions. Little, however, is known about their activity in normal cellular processes. In this study, we examined the expression pattern of eight cellular oncogenes during embryonic and fetal development of the mouse. Five of these genes (c-*myc*, c-*erb*, c-Ha-*ras*, c-*src*, and c-*sis*) were expressed at appreciable levels, and four were modulated in a consistent manner during the course of prenatal development.**

Virtually all major types of malignancy, including carcinomas, sarcomas, leukemias, and lymphomas, can be induced by one or another of the RNA tumor viruses (retroviruses) in various vertebrates (1). All of the acutely transforming retroviruses are now known to carry specific genetic information capable of inducing the malignant phenotype. These genes are designated viral oncogenes (v-oncs), and more than 16 have been identified and isolated (2–5). DNA sequences homologous to the v-onc genes have also been identified in various uninfected vertebrates and have been generically designated cellular oncogenes (c-oncs) (4, 5). Existing evidence suggests that the c-onc genes were the progenitors of the v-onc genes, a phenomenon that is believed to have occurred by recombinational events (5, 6). The fidelity with which the c-onc genes are conserved over a wide span of evolution strongly suggests that these genes serve critical physiologic functions.

A growing body of evidence indicates that at least some of the c-onc genes are expressed in normal, nontransformed cells (7–19). Given the ability of viral oncogenes to induce abnormal poliferation, it seemed appropriate to investigate the expression of their cellular counterparts (c-oncs) in physiologic cellular proliferation. One system in which to study normal proliferation *in vivo* is embryonic development. The expression of some cellular oncogenes during murine embryonic and fetal development has been described (20, 21). In the current study, we extended these observations by presenting data on expression of cellular oncogenes homologous to v-onc sequences of five avian, one feline, and one primate retrovirus.

MATERIALS AND METHODS

Murine Embryos and Fetuses. A breeding colony of white Swiss–Webster mice provided embryos. With the day of coital plug formation designated as day 0, embryos were taken at daily intervals, between 1000 and 1200 hr, from day 7 through day 18 of gestation. Pregnant female mice were killed by cervical dislocation and the uteri were immediately removed and placed in phosphate-buffered saline on ice. All subsequent manipulations were carried out on ice, and all materials for analysis were quick-frozen and stored in liquid nitrogen to ensure preservation of RNA. Uteri were opened and the embryos removed. Beginning at day 10, the embryo proper was separated from the extraembryonal membranes and placenta, and each was separately frozen. Separation was not attempted before this time because of limitations of embryo size. Day 7 to day 9 embryos therefore represent the entire conceptus as dissected from the uterine wall.

RNA Preparation and Analysis. Total RNA was extracted from whole embryos or extraembryonic tissues by homogenization in guanidine thiocyanate and precipitation with ethanol and guanidine hydrochloride as described (22). To assess the integrity of RNA to be analyzed, a 5-μg aliquot from each sample was electrophoresed in a 1.1% agarose gel and stained with ethidium bromide (0.5 μg/ml in 150 mM Tris buffer, pH 7.4). A 28S/18S ribosomal RNA ratio of less than 2:1 or fragmentation of either species was taken as evidence of degradation, and the sample was not further analyzed. The poly(A)$^+$-rich messenger RNA fraction was obtained by passage over oligo(dT) cellulose columns (23), and again a 5-μg aliquot of each sample was electrophoresed and stained. Sufficient 28S and 18S ribosomal RNA remained after one passage over oligo(dT) to again ascertain integrity of the RNA. Any sample showing evidence of significant degradation was discarded, because negative results could reflect degradation of the RNA rather than absence of transcripts in the sample.

The poly(A)$^+$ RNA was precipitated in 2.5 vol of ethanol and then suspended in water at a concentration of 2 μg/μl. The RNA was heated to 100°C for 2–3 min and quickly cooled on ice; 3 μg was spotted onto nitrocellulose paper previously equilibrated with 3.0 M NaCl/0.3 M sodium citrate (20, 24). After baking in a vacuum oven at 80°C for 3–4 hr, the blots were hybridized to one of various ^{32}P-labeled (nick-translated), molecularly cloned viral oncogene probes: v-*src*, *Pvu* II/*Pvu* II (25); v-*myc*, *Pst* I/*Pst* I (26); *erb*T (sequences representing both the *erb*A and *erb*B domains), *Pvu* II/*Pvu* II; v-*erb*A, *Pst* I/*Pst* I; v-*erb*B, *Bam*HI/*Bam*HI (27); v-*myb*, *Bam* I/*Bam* I (28); v-*mos*, *Xba* I/*Hin*dIII (29); v-Ha-*ras*, *Bgl* I/*Sal* I (30); v-*fes*, *Pst* I/*Pst* I (31); v-*sis*, *Pst* I/*Xba* I (32). To test the system for the validity of time-related and tissue-specific expression of transcripts, an α-fetoprotein (AFP)-specific probe was used as a control (33). AFP is known to be expressed in a few cells of the visceral endoderm at approximately day 7 of mouse embryonic development. Later in embryonic development it is expressed at higher levels in the fetal liver (34, 35). As shown in Fig. 1 (column 1), AFP is undetectable by dot-blot analysis in day 7 to 8 embryos, is detectable at day 9, and persists throughout subsequent development. These findings are in close agreement with published data (34, 35). Results on expression of c-*mos*- and c-Ha-*ras*-related transcripts have been published (20) and are included in these experiments as negative and positive controls, respectively. The sensitivity of the dot hy-

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Abbreviations: AFP, α-fetoprotein; kb, kilobase(s).

bridization technique was established by spotting serial dilutions of MC-29 viral RNA on nitrocellulose and subsequent hybridization to a *myc*-specific probe. The limit of detection of *myc*-specific RNA is ≈40 pg and is consistent with the range of detection published previously (20). Samples showing lesser degrees of hybridization were regarded as negative. Relative amounts of c-onc expression during mouse embryonic development were determined by soft-laser density scanning of the dot-blot autoradiograms. Estimates of the levels of onc-related RNA found at various days of embryonal development were made by spotting 4 μg of poly(A)$^+$ embryonic tissue RNA on the same filters with known amounts of 70S gradient-purified viral RNA in the case of *myc*-related sequences. Samples of embryonic tissues positive by dot-blot analysis were further characterized by RNA blot analysis (36) to confirm the presence of and to size specific transcripts.

RESULTS

Blot hybridizations using RNA from days 7–18 of embryonic development with various v-onc probes are shown in Fig. 1. Results with v-*myc*, v-*myb*, v-*src*, v-*erb*, v-*fes*, and v-*sis* (columns 4–11) are compared with existing data on the expression patterns of v-*mos*- and v-Ha-*ras*-related probes, as well as AFP, during murine embryonic and fetal development (columns 1–3) (20).

c-*myc*-related sequences were detectable by days 7 and 8 but occurred at much higher levels in late (days 17 and 18) embryonic development. Comparison of relative levels of expression by densitometric scanning of the autoradiograms showed a 3- to 5-fold increase in c-*myc*-related message at day 17 of development over the preceding 8 days and a 2-fold increase over days 7 to 8 (Fig. 2). By RNA blotting, a single transcript of ≈2.7 kilobases (kb) was easily visible at day 17, as compared with day 11 (Fig. 3).

Expression of c-*erb*-related sequences is illustrated in columns 7–9 of Fig. 1. The probe used in column 7 was representative of both *erb* domains (*erb*T). The *erb* gene consists of two individual domains, *erb*A and *erb*B (37); the results for the two separate domains are shown in columns 8 and 9. Hybridization evident with *erb*T is largely due to expression of the *erb*A component. Densitometric scanning (Fig. 2) revealed a 2- to 4-fold increase of c-*erb*-related sequences at day 14, as compared with the preceding days, and similar levels for days 14 to 18. Poly(A)$^+$ RNA from days 7 and 14 were analyzed by RNA blotting (Fig. 3), and transcripts of 2.3 kb were found in day 14 embryos and at lesser amounts at day 7.

Expression of c-*src* is illustrated in lane 6 of Fig. 1. Sequences homologous to c-*src* were detected at their highest levels in the latter half of mouse embryonic development, with an increase beginning at day 12, peaking at day 14, and gradually decreasing thereafter (Fig. 2). Agarose gel electrophoresis and RNA blotting revealed transcripts of 4.0 kb (Fig. 3).

Sequences related to v-*sis*, the transforming gene of simian sarcoma virus, are expressed throughout mouse embryonic development (Fig. 1, column 11). Two peaks were observed, one at day 7 and one at day 16. By densitometric scanning, the more prominent peak at day 7 was 1.5 to 3 times higher than those for all other days (Fig. 3). RNA blotting of day 7 and day 15 RNA showed v-*sis*-related transcripts of 3.9 kb (Fig. 3).

Embryonic tissue showed no detectable c-*myb* transcripts, although the probe used hybridized to mouse genomic DNA (Fig. 1, column 5).

RNA from days 7–18 of embryonic development showed no appreciable hybridization with the v-*fes* probe (Fig. 1, column 10).

Quantitative estimates of c-onc gene expression at various stages of development were made by spotting embryo RNA and known amounts of viral RNA. In the case of c-*myc*, 70S gradient-purified viral RNA (provided by Peter Duesberg, Berkeley, CA) was used as the standard. When 4 μg of poly(A)$^+$ RNA from day 17 embryo tissue was applied to the filters, the level of c-*myc* expression was ≈170 pg/μg of RNA (Fig. 4). When poly(A)$^+$ RNA from day 11 embryo tissue was used, the level of c-*myc* expression was ≈30 pg/μg of RNA (Fig. 4). The estimates derived by this method agree with the data obtained by densitometric analyses of the blots (i.e., the level of c-*myc*-related sequences at day 17 was 3- to 5-fold higher than that at day 11).

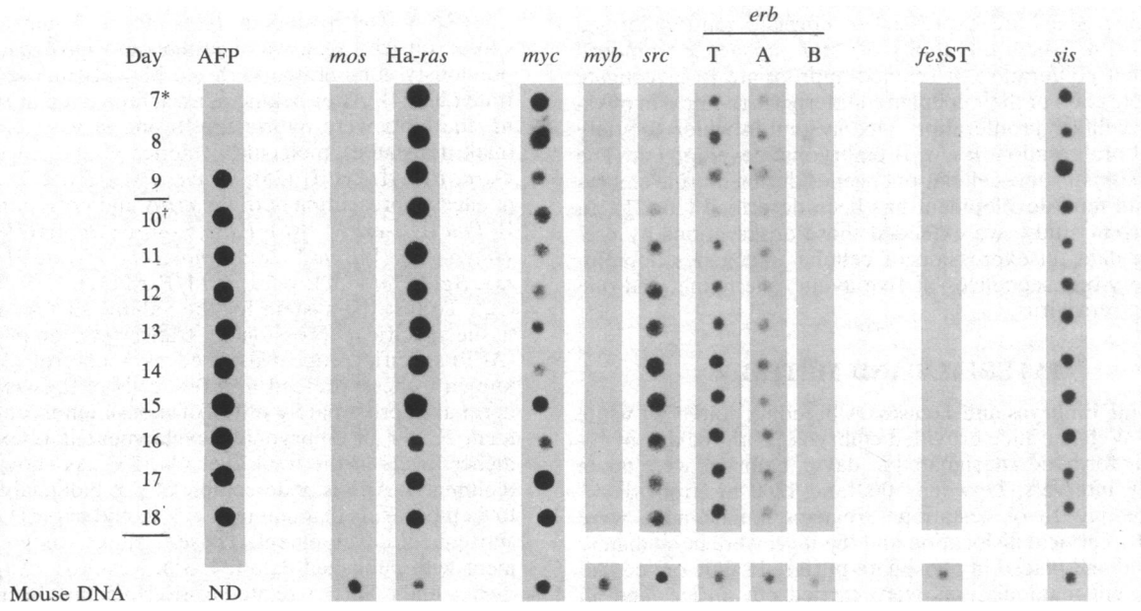

FIG. 1. Expression of cellular oncogenes during embryonic and fetal development of the mouse. ND, not done. The *erb*T probe contains sequences homologous to both *erb*A and *erb*B.
*Days 7–9, embryos and extraembryonal tissue.
†Days 10–18, embryos and fetuses.

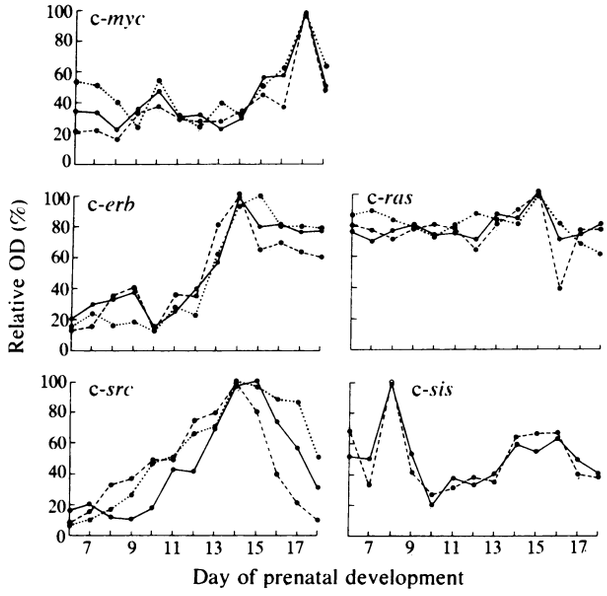

FIG. 2. Relative amounts of c-onc expression during mouse embryonic development were determined by soft-laser density scanning of the dot-blot autoradiograms shown in Fig. 1. Relative OD was plotted against day of development and values were normalized to 100, which represented the highest level of expression of a given c-onc. Scans of dot blots from three separate experiments (two in the case of c-*sis*), each representing a separate series of embryo isolates, are shown. Relative levels of c-onc gene expression are reproducible to within 24 hr from series to series.

The same technique was used in estimating the levels of c-Ha-*ras*- and c-*src*-related sequences at various stages of development, except that RNA from cells transformed with Harvey murine sarcoma virus and Rous sarcoma virus, respectively, were used as standards rather than isolated viral RNA. Assuming that the viral RNA constitutes 0.1% of the total cellular message in such cells (38, 39), the level of c-Ha-*ras*-related sequences was estimated to be 45 pg/μg in day 11 and day 18 embryonic tissue, and the level of c-*src*-related sequences was estimated to be 25 pg/μg in day 15 embryonic tissue.

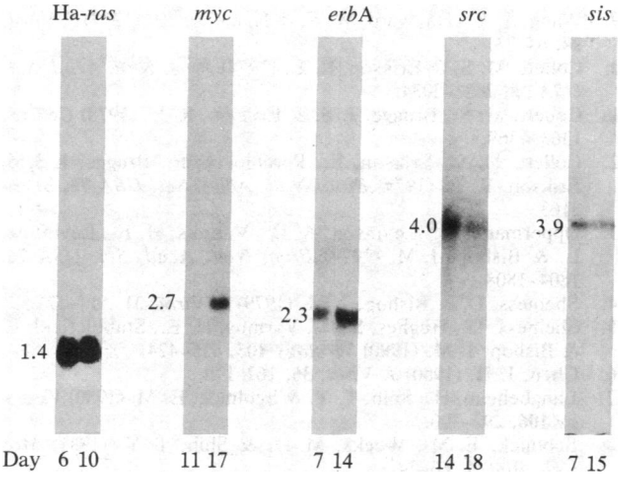

FIG. 3. Samples of embryonic tissues positive by dot-blot analysis were further characterized by RNA blot analysis (36) to confirm the presence of and to size (in kb) specific transcripts. The relative amounts of c-onc-related transcripts seen at various days were consistent with the various levels shown on dot-blot analysis. All blots were from gels containing 20 μg of poly(A)$^+$ RNA per lane.

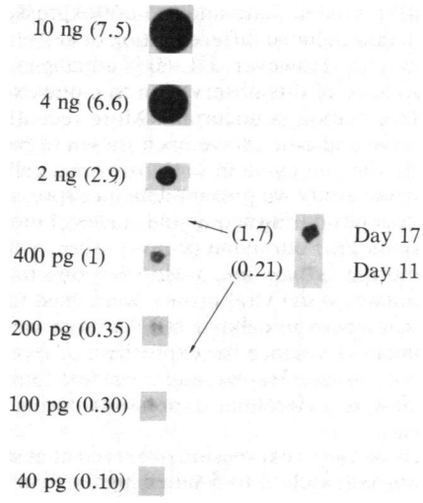

FIG. 4. The sensitivity of the dot hybridization technique was established by spotting serial dilutions of MC-29 viral RNA on nitrocellulose and subsequent hybridization to the *myc*-specific probe. The limit of detection of *myc*-specific RNA is ≈40 pg and is consistent with the range of detection previously published (20). Samples showing lesser degrees of hybridization were regarded as negative. Estimates of the levels of onc-related RNA found at various days of embryonal development was made by spotting 4 μg of poly(A)$^+$ embryonic tissue RNA on the same filters with known amounts of 70S gradient-purified viral RNA in the case of *myc*-related sequences. The dot intensity values found in embryonic tissues were normalized to 400 pg = 1 and are shown in parentheses, because this value was on the linear part of the intensity–amount curve. Thus, 4 μg of day 17 RNA had a value of 1.7, representing 680 pg of *myc*-specific RNA/4 μg of poly(A)$^+$ RNA or 170 pg/μg.

These values are only approximations of the levels of c-onc-related message found in the embryos. The method may underestimate the actual levels expressed, because the standards used share greater sequence homology with the probes than do the murine cellular homologues. In addition, it is important to note that these values represent an average level of expression in the total embryo and may not be representative of individual tissues. Expression of c-onc-related sequences at high levels by specific populations of cells at a given day of development is more likely than uniform expression in all embryonic tissues.

DISCUSSION

The structural similarities between the cellular oncogenes and their viral counterparts suggest that the c-onc genes may also possess an oncogenic potential. Evidence linking cellular oncogenes to cell transformation *in vitro* and malignant disease *in vivo* has been reviewed (2–6, 40–42). The pathologic expression of cellular oncogenes resulting in the malignant state would appear to confer no competitive advantage to organisms retaining these genes. Therefore, an argument—albeit a teleologic one—can be made that the evolutionary conservation of the cellular oncogenes indicates that they serve critical functions other than the induction of cancer. Little, however, is known about the role of cellular oncogenes in the physiology of normal cells. Evidence related to expression of two cellular oncogenes suggests that they may play a role in cellular differentiation. c-*myb* transcripts occur in various amounts in different tissues from late-stage chicken embryos and newborn chicks and appear to correlate with development of granulopoiesis in the hematopoietic system (16). c-*myb* and c-*myc* transcripts correlate with the state of cellular differentiation in the human promyelocytic leukemia cell line, HL-60 (43, 44). Both genes are expressed

in the undifferentiated state and are not expressed after dimethyl sulfoxide-induced differentiation of cells to more mature granulocytes. However, HL-60 is a malignant cell line, and the relevance of this observation to c-onc expression in normal differentiation is uncertain. More recently, two oncogenes, c-*myc* and c-*ras*, have been shown to be expressed in relation to the cell cycle in untransformed cells (45).

In the current study we present data on expression of eight cellular oncogenes during sequential stages of mouse embryonic development. Four avian (v-*myc*, v-*erb*, v-*myb*, and v-*src*), two murine (v-*mos* and v-Ha-*ras*), one feline (v-*fes*), and one primate (v-*sis*) viral probes were used to detect homologous sequences in cellular mRNA from various stages of development. Evidence for expression of five oncogenes (c-*myc*, c-*erb*, c-*src*, c-Ha-*ras*, and c-*sis*) was found, and four of these showed differential expression during embryonic development.

The levels of c-*myc* expression observed at gestational day 17 were approximately 2 to 5 times higher than those of all other days of development. Expression of c-*erb*-related sequences was observed to increase progressively from day 11 to a peak at day 14. The bulk of the expressed c-*erb*-related sequences was homologous to the *erb*A domain. Of interest is the fact that the transforming potential of the *erb* gene locus appears to reside in the *erb*B domain (46, 47). No significant expression of *erb*B was found during murine embryogenesis. c-*src*, like c-*erb*, was expressed during the latter half of fetal development.

Expression of these three genes (c-*myc*, c-*erb*, and c-*src*) varied in a consistent fashion when whole mouse embryos or fetuses were analyzed in three separate experiments. It is likely, however, that the major expression of the genes occurs in a limited cell population or tissue, rather than in all embryonic or fetal cells of a given stage. Given the difficulty in obtaining sufficient amounts of individual embryonic tissues (i.e., spleen, muscle, liver, brain), techniques that will allow localization of transcripts or translation products in whole embryo and fetal sections may prove useful in localizing the tissues responsible for the time-related expression of these oncogenes. Such an approach has been successfully used to demonstrate developmental regulation of c-*src* in the neural retina of chicken embryos (48).

Unlike c-*myc*, c-*erb*, and c-*src*, c-Ha-*ras* and c-*sis* were expressed throughout mouse embryonic development. It has been shown previously that c-*ras* expression is not restricted to tissues of the embryo but occurs at similar levels in placenta and extraembryonal membranes (20). Speculation concerning assignment of c-*ras* expression to a specific tissue or stage of development is difficult because of its ubiquitous presence. This finding may indicate a role for c-*ras* in many different proliferating or differentiating cells.

Recent evidence has tied the putative transforming protein encoded by v-*sis* to a normal growth factor, platelet-derived growth factor (49, 50). The expression of c-*sis*-related sequences as reported here may reflect the presence of the same or a similar growth factor during murine development.

Although sequences homologous to v-*myb*, v-*mos*, and v-*fes* could be detected in mouse DNA, no appreciable transcriptional expression of these cellular genes was found in embryos. These data, however, should be viewed in the context of the sensitivity of the methodology used. It may be that these genes are expressed but at levels below our limits of detection. This might be particularly true if this expression is restricted to a limited cell population.

In addition, there is variability of degree of hybridization of the v-onc probes to the c-onc homologue found in murine DNA (Fig. 1). However, there appears to be no consistent correlation between the degree of DNA hybridization and the ability of the probe to detect transcripts. Some probes that show strong hybridization to murine DNA do not detect transcripts (e.g., v-*mos*), and some that hybridize to a lesser degree to DNA readily detect transcripts (e.g., v-Ha-*ras*).

The current study indicates that some c-onc genes are expressed in mouse embryonic and fetal development. In parallel studies, stage-specific and tissue-related expression of c-*abl*, c-*fos*, c-*fms*, and c-Ki-*ras* during murine development has been found (20, 21). Thus, evidence from this and other studies points toward a role for these genes in normal cellular proliferation and/or differentiation. The relatedness of the v-*sis* gene product to a normal cellular growth factor (platelet-derived growth factor) underscores this concept. More recently, another oncogene, c-*erb*B, has been shown to be related to the epithelial growth factor receptor (51). Further studies of the structure of other growth factors or their receptors may reveal similarities to still other cellular oncogenes. In addition, two cellular oncogenes, c-*myc* and c-Ha-*ras*, are expressed in a temporal relationship to the cell cycle in untransformed cells (45), giving further credence to the idea that these genes play a role in the process of normal cell growth. Thus the term cellular oncogene may not be wholly appropriate in that the physiologic function and not the transforming ability of these genes most likely accounts for their avid conservation. An association of cellular oncogenes with the malignant state may merely reflect the untimely or inappropriate expression of genes whose functions are central to normal growth and development.

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