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Two proto-oncogenes implicated in mammary carcinogenesis, *int-1* and *int-2*, are independently regulated during mouse development

(oncogenes/mouse mammary tumor virus/embryos/teratocarcinoma cells/testes)

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ABSTRACT **The *int-1* and *int-2* genes were first isolated as targets for transcriptional activation by proviral insertion mutations in mammary carcinomas induced by the mouse mammary tumor virus (MMTV). Since these proto-oncogenes are not expressed at detectable levels in previously tested normal tissues from adult mice, we sought to determine whether these genes might be active during embryogenesis by examining mouse embryos and cultured teratocarcinoma cells for RNA encoded by *int-1* and *int-2*. A single size class of *int-1* RNA is present only at mid-gestation stages of development (days 8 through 13) and is also detected in testes from postpuberal mice. Four species of *int-2* RNA are found in peri-implantation embryos and teratocarcinoma cells and are particularly abundant in derivatives of the primitive endodermal lineage, but *int-2* RNA is not detected during mid- or late gestation or in any normal adult tissues tested. Thus, these two proto-oncogenes, activated during mammary carcinogenesis by the same mechanisms, are normally expressed at different times and places in embryonic and adult mice.**

The normal functions of proto-oncogenes, the evolutionarily conserved progenitors of mutant genes that contribute to neoplastic change, are widely thought to affect growth and development (1). This hypothesis has gained support from several experimental observations: some proto-oncogenes have been identified as genes encoding known growth factors or growth factor receptors; the products of some proto-oncogenes have been implicated in the transition of cultured cells from a resting to a growing state; mutant forms of some proto-oncogenes can arrest, distort, or promote differentiation; and expression of certain proto-oncogenes fluctuates temporally or spatially during development in vertebrate and other metazoan animals (see ref. 2 for review).

Most evidence of this sort has been gathered through study of proto-oncogenes that are cellular progenitors of retroviral oncogenes (c-*onc* genes) or close relatives of c-*onc* genes. The work reported here concerns two recently discovered proto-oncogenes, *int-1* and *int-2*, which have not been encountered among the oncogenes transduced by retroviruses and display no evident homology with known c-*onc* genes or each other. These genes were first identified as mouse genomic sequences adjacent to integrated proviruses of the mouse mammary tumor virus (MMTV) in a large proportion of virus-induced tumors in certain mouse strains (3, 4). As a consequence of enhancer elements in the inserted provirus, abnormal expression of the adjacent gene is provoked, and this presumably contributes to the formation of a mammary carcinoma. Tumors with proviral insertions in *int-1* or *int-2* contain several copies per cell of polyadenylylated RNA from the affected gene, but *int-1* and *int-2* encoded RNAs are undetectable in nonneoplastic mammary tissue and in a variety of other normal tissues and cells, and the genes have not been implicated in tumorigenesis in other settings (5, 6). These findings suggest that *int-1* and *int-2* are infrequently expressed in normal cells and perhaps tissue-specific in their neoplastic effects. Such characteristics might be indicative of genes that function in narrow developmental windows as, for example, in specific cell types or at defined times in the developing embryo. The study described here was undertaken to explore such possibilities by surveying for *int-1* and *int-2* RNA during embryonic development.

MATERIALS AND METHODS

Preparation of RNA from Embryos, Adult Organs, and Cultured Cells. Embryos were obtained by mating mice of various strains. The day on which the vaginal plug was detected was termed day 0.5 of gestation. Embryonic, placental, and yolk sac samples were obtained from 10 to 100 conceptuses, depending on gestational age; organs were dissected from adult mice of various strains. Total cellular and poly(A)$^+$ RNA were extracted as described by Joyner *et al.* (7).

PSA1 teratocarcinoma stem cell cultures were maintained in the undifferentiated state by coculture with STO fibroblastic feeder cells and were induced to differentiate to form embryoid bodies, as described (8). Stage 1 and stage 2 embryoid bodies were collected after 9 and 17 days, respectively, of culture in bacteriological dishes. For stage 3 cultures, stage 2 embryoid bodies were seeded in tissue culture dishes and cultured for an additional 7 days. Purified populations of PSA1-derived endodermal cells were obtained from the embryoid bodies that had formed after 6 days of culture in bacteriological dishes according to the procedure described by Grabel and Martin (9).

F9 embryonal carcinoma cells were maintained in the undifferentiated state or stimulated to form endoderm by incubating cultures of undifferentiated cells with 0.2 μM retinoic acid and 1 mM dibutyryl cyclic AMP (Bt_2cAMP) for 4 days (10). PYS and STO cells were cultured as previously described by Lehman *et al.* (11) and by Martin and Evans (12), respectively. Total cytoplasmic RNA was extracted from the different cell types as described by Jakobovits *et al.* (13).

Mammary Tumor RNA. Poly(A)$^+$ RNA from mammary tumor U153 (14) was kindly supplied by G. Peters and C. Dickson (Imperial Cancer Research Fund, London). Poly(A)$^+$ RNA from a C3H mammary tumor with an MMTV provirus 5′ of the *int-1* gene was prepared as described by Fung *et al.* (15).

Electrophoretic Analysis of RNA. Total or poly(A)$^+$ RNAs were fractionated by electrophoresis in a 1% agarose/formaldehyde gel, and the RNA species were transferred to

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Abbreviations: MMTV, mouse mammary tumor virus; EC cells, embryonal carcinoma cells; ICM, inner cell mass; bp, base pair(s); kb, kilobase(s).

GeneScreen nylon membranes (13). The RNA was then cross-linked to the membrane by ultraviolet irradiation, using the procedure of Church and Gilbert (16). ^{32}P-Labeled double-stranded or single-stranded probes were hybridized to the membranes as described (13).

Molecular Clones and Hybridization Probes. The *int-1* probe was a 1.65-kilobase (kb) fragment extending from the 5′ end of cDNA clone 26 to the *Stu* I site in the last exon (15). The *int-2* probe was a 0.6-kb *Bam*HI–*Eco*RI genomic fragment which encompasses probe *f* described by Moore *et al.* (17). The chicken β-actin clone was kindly provided by D. Cleveland (18). Double-stranded nick-translated probes were made by labeling gel-purified DNA fragments by standard procedures (19). Single-stranded *int-2* probes were synthesized by primer extension on M13 phage DNA by described methods (20).

Copy Number Estimations. The intensities of autoradiographic signals on RNA blots from tumor and embryo or F9-endodermal cells were compared with those of defined amounts of *int-1* or *int-2* encoded RNAs synthesized by SP6 RNA polymerase (21) from plasmid templates containing the *int-1* or *int-2* probe fragments. Concentrations of these synthetic RNAs were determined by measurement of absorbance (at A_{260}) of the Sephadex G-50 void fraction, after subtraction of the A_{260} absorbance of a reaction mixture without polymerase. Copy numbers were calculated assuming that an average cell contains 10^{-5} μg of RNA, that poly(A)$^+$ RNA represents about 3% of total cellular RNA, and that the average mRNA is 2 kb in length (19).

RESULTS

Expression of *int-1* and *int-2* in Embryos. Mouse embryos were collected at daily intervals from days 7.5 to 17.5 of gestation to assay for *int-1* and *int-2* RNA species during development. Only embryonic tissue was included in each sample with the exception of the day 7.5 sample, which contained the embryo proper and adjacent extraembryonic tissue but not parietal endoderm or ectoplacental cone. In addition, the placenta and visceral yolk sac (extraembryonic tissues) were isolated from conceptuses at day 11.5 of gestation. Total cellular RNA was isolated from samples harvested between day 7.5 and day 9.5 of gestation, and poly(A)$^+$ RNA was prepared from the samples taken between day 9.5 and day 17.5.

Independent temporal patterns of expression for the two genes were observed (Fig. 1) following fractionation of the RNA species by gel electrophoresis, RNA transfer, and hybridization of ^{32}P-labeled probes that represent all or part of the coding portions of *int-1* and *int-2* genes, respectively. *int-1* RNA was not detectable in samples from day 7.5 of gestation, but a species ≈2.6 kb in size appeared in the day 8.5 samples, was found at maximum levels in day 9.5–11.5 samples, and was again undetectable by day 13.5–14.5 of gestation. No *int-1* RNA was detected in placenta or visceral yolk sac. In contrast, *int-2* RNA was undetectable in all samples, except the day 7.5 RNA, in which four classes of *int-2* RNA—3.2, 2.9, 1.8, and 1.4 kb—were observed. Subsequent hybridization with β-actin probe showed that appropriate amounts of intact RNA were present in the lanes on this filter (data not shown). Thus, the *int-1* gene is expressed in the embryo during the mid-gestation period, from approximately 8 to 13 days of development, whereas *int-2* is expressed only at the earliest time tested, day 7.5.

Expression in Teratocarcinoma Cells. Since levels of *int-2* RNA are relatively high in the day 7.5 sample in Fig. 1, it seemed likely that the gene is also expressed at earlier times when it is difficult to obtain sufficient tissue for analysis. To investigate this possibility for *int-2*, as well as for *int-1*, we extended our survey to include teratocarcinoma cells at various stages of differentiation, which serve as an *in vitro* model for embryonic cells at early stages of development (22). The data to be described are best appreciated in the context of knowledge of the differentiation that occurs during the peri-implantation period of embryogenesis (Fig. 2) and the way in which these events are mimicked by teratocarcinoma cells.

At approximately day 3.5 of gestation the pluripotent portion of the mouse embryo consists of a group of cells known as the inner cell mass (ICM). Shortly before implantation, at approximately day 4.5 of gestation, the outer cells of the ICM differentiate to primitive endoderm, and the remaining cells form the embryonic ectoderm, from which the fetus will be derived. The descendants of the primitive endodermal cells never form part of the embryo proper, but instead become either the parietal endoderm or the endodermal layer of the visceral yolk sac, both extraembryonic portions of the conceptus (23). Undifferentiated teratocarcinoma stem cells (embryonal carcinoma cells; EC cells) are thought to be equivalent to ICM cells prior to endoderm formation. Following the formation of free-floating rounded cell aggregates, certain EC cell lines such as PSA1 differentiate *in vitro* in a manner that closely mimics the peri-implantation development of the embryo (8, 12, 22). At

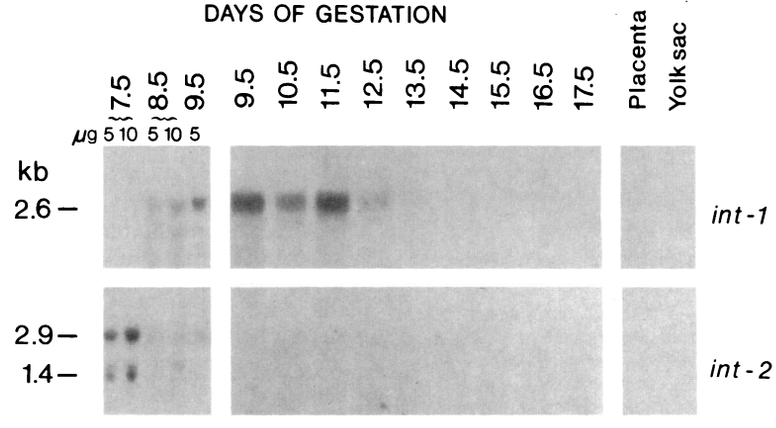

FIG. 1. Detection of *int-1* and *int-2* encoded RNA in mouse embryos. RNA samples were subjected to electrophoresis in formaldehyde/agarose gels, transferred to nylon sheets (GeneScreen), and hybridized with ^{32}P-labeled probes for *int-1* (*Upper*) or *int-2* (*Lower*), as described in *Materials and Methods*. From left to right: unfractionated RNA (5 or 10 μg) from the embryo (including the extraembryonic ectoderm and proximal endoderm) at day 7.5 of gestation and from embryos at days 8.5 and 9.5 of gestation; poly(A)$^+$ RNA (5 μg) from fetuses at days 9.5–17.5 of gestation and from placenta and visceral yolk sac dissected from the conceptus at day 11.5 of gestation. The sizes of indicated RNA species were determined from positions of stained bands of ribosomal RNA.

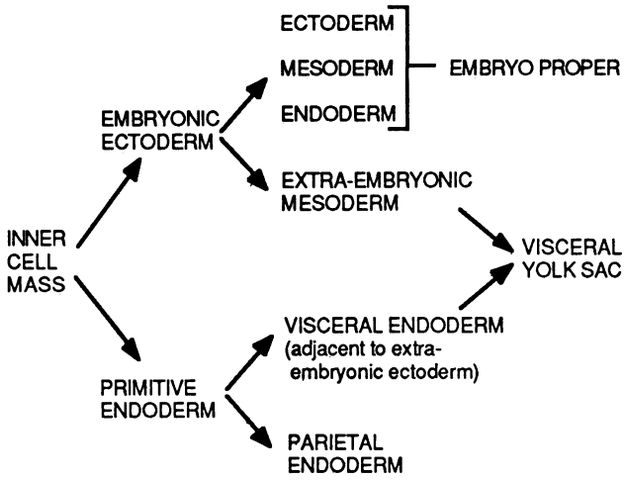

FIG. 2. Schematic representation of ICM-derived cell lineages in the peri-implantation mouse embryo (see ref. 23 for details).

stage 1 in their differentiation, PSA1 cell aggregates, known as embryoid bodies, consist of a stem cell core and an outer layer of endoderm and are analogous to the embryo at approximately day 4.5–6.5 of gestation; at stage 2, PSA1 embryoid bodies contain cell types present in embryos at day 6.5–8.5 of gestation as well as a structure that resembles the visceral yolk sac (8); stage 3 cultures, obtained by allowing the embryoid bodies to attach to a substratum, resemble mid-gestation embryos in that they contain a heterogeneous cell population including keratinizing epithelium, cartilage, striated muscle, and nerve (24).

When RNA from PSA1 cells harvested at various stages of differentiation was examined for *int-1* species (Fig. 3), low levels of the 2.6-kb transcript were detected in stage 2 and 3 cultures, but not in stage 1 embryoid bodies, in an endodermal cell population derived from embryoid bodies, or

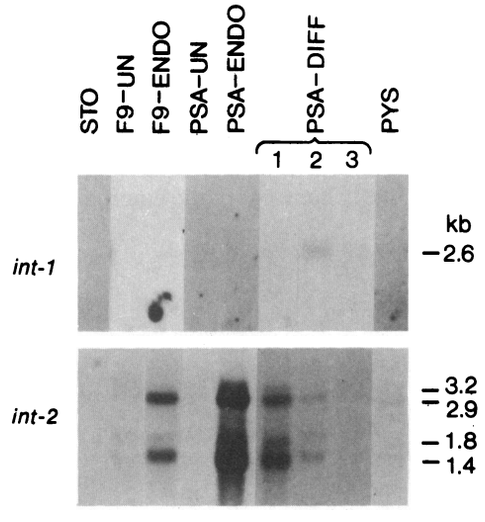

FIG. 3. Expression of *int-1* and *int-2* RNA in teratocarcinoma cells. Poly(A)$^+$ RNA from the indicated sources was analyzed as described in the Fig. 1 legend for *int-1* (*Upper*) and *int-2* RNA (*Lower*). From left to right: STO cells, an established mouse embryo fibroblast cell line; undifferentiated F9 teratocarcinoma stem cells; F9-derived endodermal cells; undifferentiated PSA1 teratocarcinoma stem cells; PSA1-derived endodermal cells; PSA1 cultures that had been stimulated to differentiate to stage 1 (simple embryoid bodies), stage 2 (cystic embryoid bodies), or stage 3 (heterogeneous cultures of cells from attached embryoid bodies); PYS cells, an established parietal endoderm-like cell line. Each lane contains ≈5 μg of RNA, except for the PSA-ENDO lane which contains ≈15 μg.

in undifferentiated cells. *int-1* RNA was also undetectable in undifferentiated F9 EC cells, in F9 cell monolayers induced to form parietal endoderm-like cells by treatment with retinoic acid and Bt_2cAMP (10), and in PYS cells, an endodermal cell line derived from teratocarcinoma embryoid bodies and endowed with many of the properties of parietal endoderm (11). In addition, no *int-1* RNA was detected in STO fibroblasts, with which PSA1 cells are cocultured in order to maintain them in the undifferentiated state (12). Since the stage 2 and stage 3 PSA1 cultures in which *int-1* RNA was detected contain some cell types found in embryos at approximately day 8 of gestation or thereafter, these data are consistent with our findings in intact embryos (Fig. 1); moreover, the absence of detectable *int-1* RNA in the other teratocarcinoma cell samples suggests that the *int-1* gene is not expressed in embryos prior to day 6.5 of gestation.

More dramatic results were observed when the same samples were examined with the *int-2* probe (Fig. 3): the four species of RNA previously seen in the day 7.5 embryo sample in Fig. 1 were found in several of the samples, particularly those enriched in cells of the primitive endodermal lineage. Relatively low and approximately equal levels of four species of *int-2* RNA, 3.2, 2.9, 1.8, and 1.4 kb in length, are present in undifferentiated PSA1 cells. Much higher levels, particularly of the 2.9- and 1.4-kb species, are present in PSA1 stage 1 embryoid bodies [in which the endodermal cells have features of either parietal or visceral endoderm, or both (8)] and in an endodermal cell population isolated from the embryoid bodies (PSA-ENDO). The latter result suggests that the endodermal cells are partially or wholly responsible for the levels of *int-2* RNA observed in the unfractionated embryoid bodies. Progressively lower levels of *int-2* RNA were detected in stage 2 and stage 3 embryoid bodies, suggesting that the levels of *int-2* RNA decrease either when a large proportion of endodermal cells in the embryoid bodies develop into visceral yolk sac endoderm (stage 2) or when only a small fraction of the total cell population is derived from the primitive endodermal lineage (stage 3).

Similar results were obtained during differentiation of the F9 EC cell line. Low levels of the four species of *int-2* RNA are present in undifferentiated F9 cells, but the abundance of the 2.9- and 1.4-kb species is markedly increased in F9 cells induced to differentiate into endoderm. In PYS cells the levels of *int-2* RNA are lower than in F9-derived endodermal cells, but the overall pattern is the same, with the 2.9- and 1.4-kb species predominating. No *int-2* RNA was detected in STO fibroblasts. Taken in concert, the findings suggest that production of *int-2* RNA, particularly the 2.9- and 1.4-kb transcripts, is characteristic of the primitive endodermal lineage, because expression is observed (*i*) in cells resembling parietal endoderm (F9 endoderm and PYS), (*ii*) in tissue containing visceral endoderm (yolk sac precursors) but not parietal endoderm (day 7.5 embryo), and (*iii*) in cells with features of both endodermal types (PSA stage 1). The absence of *int-2* RNA in the visceral yolk sac of day 11.5 conceptuses argues that *int-2* expression ceases once cells in the visceral branch of the endodermal lineage undergo terminal differentiation.

Expression in Adult Tissues. Successful detection of *int-1* and *int-2* RNA in developing embryos prompted a wider search for adult organs in which these genes are active. The surveyed tissues included organs composed largely of epithelial cells (liver, kidney, and mammary gland), neural tissue (brain), muscle (heart, leg muscle), a hematopoietic organ (spleen), and a reproductive organ rich in germinal cells (testes) (Fig. 4 and data not shown). RNA prepared from most of these tissues did not contain detectable *int-1* or *int-2* species, as was the case in previous studies of mammary glands from pregnant and lactating animals and of spleen and liver (refs. 3, 5, and 6; Y. K. T. Fung and H.E.V., unpub-

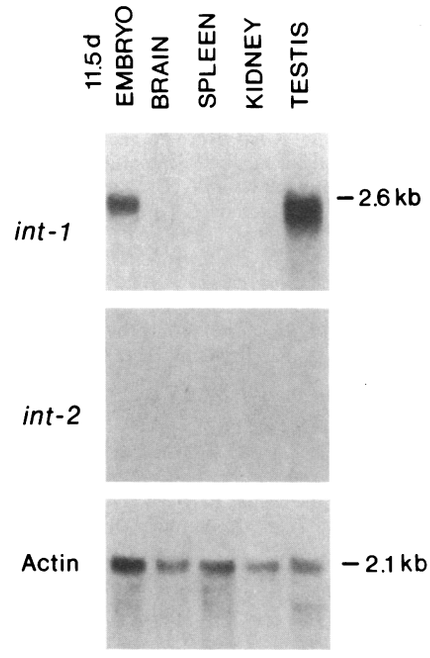

FIG. 4. Detection of *int-1* and *int-2* RNA in adult mouse tissues. Poly(A)$^+$ from the indicated sources was analyzed as described in the legend to Fig. 1 for *int-1* (*Upper*), *int-2* (*Middle*), and β-actin RNA (*Lower*). Each lane contains ≈10 μg of RNA.

lished observations); the use of a probe for β-actin again confirmed the presence of intact RNA in each lane (Fig. 4 *Lower*). One notable exception to the general absence of *int-1* and *int-2* RNA was observed; *int-1* RNA is at least as abundant in poly(A)$^+$ RNA from adult testis as in RNA from day 11.5 embryos (Fig. 4). Though similar in size in the two samples, the *int-1* RNA in testicular RNA is comparatively heterogeneous, suggesting multiple closely spaced initiation sites or variable processing. *int-1* RNA was not detectable in samples obtained from testes prior to 21 days of age (data not shown), implying that sexual maturation is required for expression.

Comparison of *int-1* and *int-2* Transcripts in Normal Cells and Tumors. To help evaluate the magnitude and manner of expression of *int-1* and *int-2* in embryonic tissues and cultured EC cells, we have compared RNA transcripts of each gene in such cells with transcripts in mammary tumors and with known amounts of RNAs synthesized *in vitro* from plasmids containing *int-1* and *int-2* (Fig. 5 and data not shown). The detected species of *int-1* RNA from embryo and tumor samples are indistinguishable in mobility, suggesting that similar or identical sites are used for initiation and processing of *int-1* RNA in the two tissues. We estimate from comparisons of autoradiographic signals that an average of 0.5 copy per cell is present in whole embryos and this mammary tumor (see *Materials and Methods*). Since we do not know the proportion of cells expressing the gene in each context, it is premature to comment upon relative efficiencies of expression.

At least two of the observed *int-2* RNA species in cells such as F9 endoderm are smaller than species observed in most MMTV-induced mammary tumors with *int-2* insertion mutations (6, 14). However, these two species, 1.8 and 1.4 kb in length, are detected in RNA from at least 10–20% of such tumors, including tumor U153, which is analyzed in Fig. 5 (G. Peters and C. Dickson, personal communication). The four major species of *int-2* RNA in the endodermal and tumor U153 RNA samples appear to comigrate, within the limits of resolution of these gels. [Two additional species of *int-2* RNA, ≈3.9 and 4.3 kb in length, are present in tumor U153;

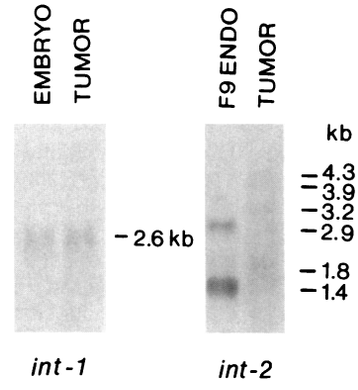

FIG. 5. Comparative sizes of *int-1* and *int-2* RNAs in embryos or teratocarcinoma cells and in mammary tumors. RNA from the indicated sources was analyzed as described in the legend to Fig. 1 for *int-1* (*Left*) or *int-2* (*Right*). *Left*: Poly(A)$^+$ RNA (5 μg each) from day 11.5 embryos and a mouse mammary tumor containing *int-1* RNA. *Right*: Poly(A)$^+$ RNA from F9 endodermal cells (2 μg) and a mouse mammary tumor (U153) containing *int-2* RNA (3 μg). The *int-2* probe used was strand-specific, produced as described in *Materials and Methods*.

these result from readthrough of the final exon of *int-2* and polyadenylylation within proviral DNA inserted ≈1 kb 3′ of the gene (C. Dickson and G. Peters, personal communication).] The amount of *int-2* RNA is estimated to be about 2 copies per cell in F9-derived endodermal cells and about 0.3 copy per cell in the tumor. Because a strand-specific *int-2* probe was used in Fig. 5, all the detected transcripts must originate from the same strand.

DISCUSSION

The *int-1* and *int-2* proto-oncogenes were discovered as genes that respond similarly to MMTV proviral insertion mutations during induction of mammary carcinomas, but there are several indications that they might have different functions: their nucleotide sequences and the deduced amino acid sequences of their protein products are unrelated to each other or to any other isolated genes or proteins (15, 17, 25), and they reside on different chromosomes (5, 26). Moreover, both of the genes are often insertionally activated in clonal mammary tumors in BR6 mice, suggesting complementary roles in tumorigenesis (14).

The data reported here are consistent with the hypothesis that the two genes are functionally distinct, since we have unequivocally distinguished them according to the times and places they are expressed during normal mouse development and in the adult. *int-1* RNA is found only in the mid-gestation fetus from approximately day 8–13 of development and in postpuberal testes, and *int-2* RNA is present prior to day 7.5 of gestation, most abundantly in cells of the primitive endodermal lineage.

Surveys of embryonic materials for expression of proto-oncogenes have been commonly employed to explore the general proposal that these genes are important in developmental programs. In mammalian embryos varied patterns of expression for different proto-oncogenes have been detected. For example, mouse embryos assayed from a few days after implantation to a few days before birth have been reported to show no detectable RNA from several proto-oncogenes, including c-*mos*, c-*myb*, c-*erbB*, and c-*fes*, and persistent expression of several others, including c-Ha-*ras*, c-Ki-*ras*, c-*myc*, and c-*sis*, at relatively constant levels (27, 28). A few proto-oncogenes are expressed at significantly elevated levels at specific stages of development. For example, N-*myc* and p53 appear to be highly expressed in the embryo from implantation through the mid-gestation period (13, 29),

whereas c-*src* is expressed at elevated levels in the latter half of the gestation period (28); c-*fos* and c-*fms* are highly expressed in the extraembryonic membranes of mouse and human conceptuses (27, 30, 31). Our data, indicating that expression of *int-1* and *int-2* is restricted to short periods of embryogenesis during which key developmental decisions are being made, suggest that they are among the most promising candidates to date for genes that play pivotal roles in mammalian development.

Although we have established that the *int-1* gene is active in the mid-gestation period, we have not defined the major site(s) of expression, nor have we excluded the possibility that the timing of expression is different in different tissues. Further studies employing *in situ* RNA hybridization assays will be required to rigorously define the sites of production in the embryo. In the adult, our survey of tissues has confirmed preliminary indications that *int-1* and *int-2* are parsimoniously expressed in mature animals. However, *int-1* is expressed in testes, as are several other proto-oncogenes. Most notably, expression of c-*mos*, like *int-1*, has been detected in adults only in the testis (32). The observations of atypically sized transcripts of c-*mos* (32) and c-*abl* (33), like the apparently heterogeneous transcripts of *int-1*, suggest that synthesis or processing of some RNAs may be significantly different in germinal and somatic tissues. *int-1* RNA is present in seminiferous tubules, only after sexual maturation (unpublished observations), implying that the gene may be expressed late in the spermatogenic cell lineage, perhaps in postmeiotic cells, as is the case for the atypical c-*abl* transcript (34).

For *int-2*, we have not only demonstrated that its expression is specific to the peri-implantation stages of development, the period during which the most fundamental decisions about cell lineage are made, but have also gained some insight into its cell type specificity. The data from studies of EC cells and their differentiated derivatives suggest that four *int-2* RNA species are expressed at low levels in ICM cells and that a dramatic increase in the levels of two of these transcripts is associated with the formation of cells in both the parietal and visceral branches of the endoderm lineage, although they are not expressed in fully differentiated visceral yolk sac endoderm. Further studies, involving localization of *int-2* transcripts in sections of mouse conceptuses, are required to fully define the sites of *int-2* production in the peri-implantation embryo. However, from the available data the stage-specificity of *int-2* expression appears unique. None of the genes known to be expressed in the precursors of the visceral yolk sac endoderm is subsequently silent in fully differentiated derivatives (35), which may signify that this gene plays some role in the process of endodermal differentiation.

The physiological functions and biochemical properties of *int-1* and *int-2* proteins are still poorly defined, precluding detailed interpretation of the patterns of expression observed here. Further characterization and localization of the RNAs and protein products of the genes in cultured cells, mammary tumors, developing embryos, and testes will now be required to help elucidate their normal and oncogenic functions.

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