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Biochemistry

Temporal and tissue-specific expression of mouse *ets* genes

(proto-*ets* gene/cell proliferation/liver regeneration/superinduction)

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ABSTRACT **The expression of *ets* genes has been studied in mouse tissues and regenerating murine liver, an *in vivo* model for cell proliferation. Our results indicate that (*i*) the *ets-1* and *ets-2* loci are transcriptionally active; (*ii*) the *ets-2* locus encodes a major mRNA (3.5 kilobases) and is expressed in most of the tissues examined, whereas the *ets-1* locus encodes a major 5.3-kilobase and minor 4.0-, 2.5-, and 2.2-kilobase RNA species and is expressed at a high level in thymus; (*iii*) both ets-1 and ets-2 mRNA are abundant in young proliferating tissues and are greatly reduced in terminally differentiated tissues, except thymus; (*iv*) compensatory growth of liver induces ets-2 mRNA before DNA synthesis, but after *fos* and *myc* induction; and (*v*) ets-2 mRNA, but not ets-1 mRNA, is stabilized in the presence of cycloheximide during hepatic regeneration. These results suggest that *ets-2* gene expression is intrinsically linked with cell proliferation. Thus, *ets-2* expression follows a pattern similar to other members of the nuclear oncogene family. During hepatic regeneration, the *ets-1* and *ets-2* loci are subject to differential regulation.**

Retroviral oncogenes are derived from normal cellular genes that may be actively involved in cell proliferation and differentiation. Avian erythroblastosis virus, E26, is a replication-defective virus that contains tripartite oncogenes Δ*gag-mybE-ets-*Δ*env* (1, 2). We have previously shown (3, 4) that (*i*) v-*ets* has two cellular homologues, *ets-1* and *ets-2*, localized on two different chromosomes in higher mammals; (*ii*) the *ets-1* locus has been mapped to human chromosome 11, mouse chromosome 9, and feline chromosome D1; the *ets-2* locus has been assigned to human chromosome 21, mouse chromosome 16, and feline chromosome C2; (*iii*) both human *ets-1* and *ets-2* loci are transcriptionally active; and (*iv*) in acute human leukemia, the *ets-1* locus has been translocated from chromosome 11 to 4 in t(4;11) (q21;23) region, whereas the *ets-2* locus has been translocated from chromosome 21 to chromosome 8 in t(8;21) (q22;23), indicating *ets* gene products may play a significant role in leukemogenesis (refs. 5–8; for a review, see ref. 8). In contrast to the mammalian genes, the chicken proto-*ets* locus appears to be contiguous and encodes a major transcript of 7.5 kilobases (kb) (2, 3).

Using temperature-sensitive mutants of E26 virus, Beug *et al.* (9) have shown that the v-*ets* domain of the E26 virus retains the capacity to transform erythroblasts and fibroblasts *in vitro*. From these results, it has been concluded that products derived from the *ets* gene loci are involved in erythroid differentiation. However, as indicated, the *ets* loci in higher mammals are very complex. We do not know how many polypeptides are encoded by the *ets* gene loci and what function they perform in the cell. To understand the biological function and regulation of the *ets* genes in higher eukaryotes, we have studied the expression of the *ets* gene loci in various murine tissues and in liver following partial hepatectomy. Our results indicate that *ets-2* gene expression is linked to cell proliferation and occurs well before DNA synthesis; and the induction of ets-2 mRNA occurs subsequent to fos and myc mRNA expression. During liver regeneration, and in the absence of protein synthesis, superinduction of ets-2 mRNA and not ets-1, occurs, indicating that the *ets* gene loci are subject to differential regulation.

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MATERIALS AND METHODS

Partial Hepatectomy and RNA Blot Analysis. Adult BALB/c male mice (5 to 6 weeks old), weighing ≈30 g, were subjected to sham or partial (removal of two-thirds of liver mass) hepatectomy (under anesthesia) following the procedure of Higgins and Anderson (10). At the indicated times (see Figs. 3–5) animals were sacrificed and total RNA was isolated from their livers by the guanidinium isothiocyanate/CsCl method (11). DNA synthesis was measured (during compensatory growth of liver) by the incorporation of [^{3}H]thymidine into DNA (12).

Tissues (2 g) were harvested from 5- to 10-day-old "young" and from 5- to 6-week-old "adult" mice. Total RNA was isolated as described above. Poly(A)$^+$ RNA isolation, size fractionation of RNA on formaldehyde-agarose (1%) gels, transfer of RNA from gel to Nytran membrane using 20× SSPE, and fixing RNA by baking at 80°C *in vacuo* for 3–4 hr, were done essentially as described in the cloning manual (13). Filters were prehybridized for 2–4 hr or overnight at 42°C [hybridization buffer: 50% formamide/5× Denhardt's solution (1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/5× SSPE/0.1% $NaDodSO_4$] and then hybridized with ^{32}P-labeled probes (0.25 ml of hybridization buffer per cm^2 containing 2–3 × 10^6 cpm/ml) for 60–72 hr. Filters were washed under stringent conditions (0.1× SSPE/0.1% $NaDodSO_4$ at 50°C) and autoradiographed for a time appropriate to detect specific transcripts. Only the relevant portions of the autoradiograms are shown.

Molecular Weight of *ets* Transcripts. The size of *ets* transcripts was determined by the mobility of standard RNA molecular weight markers (Bethesda Research Laboratories RNA ladder, and from 28S and 18S rRNA) and from the sizes of known mRNA detected on the autoradiogram. We estimate the size of murine ets-2 mRNA to be 3.5 ± 0.3 kb and of ets-1 mRNA to be 5.3 ± 0.2 kb, 4.0 ± 0.3 kb, 2.5 ± 0.2, and 2.2 ± 0.1 kb.

Preparation of Probes. One microgram of plasmid DNA or 500 ng of DNA fragment was labeled using Amersham's nick-translation kit. Free nucleotides were removed by mini-spin column chromatography (13). Only those probes having a specific activity of >10^8 cpm per μg of DNA were used to detect specific transcripts.

Probes. The following plasmid DNA or DNA fragments were used to detect transcripts by RNA blot analysis. Human *ets-1* probe pRD700: 830-base-pair (bp) *Eco*RI fragment (3);

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mouse *ets-2* probe: 1300-bp *Pst* I fragment (D. K. Watson, personal communication); α-tubulin probe: 1400-bp *Hin*dIII fragment (14); β-actin probe (15); *fos* probe (obtained from Oncor); ornithine decarboxylase probe (16); v-Ha-*ras* probe (17); heat shock protein probe (18); mouse metallothionein probe: 1100-bp *Bgl* II/*Pst* I fragment (19); and human *MYC* probe: 6000-bp *Eco*RI/*Bam*HI fragment containing all three exons (ref. 20; unpublished data).

Immunoblot Analysis. Tissues (50 mg) from young and adult mice were solubilized in 500 μl of sample buffer (21). Proteins were size-fractionated by $NaDodSO_4$/polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane (22). The blots were processed for immunoblot analysis (23) using polyclonal antisera raised against oligopeptide derived from the *ets-2* coding region (unpublished data). The antibody-bound antigens were detected by using ^{125}I-labeled protein A followed by autoradiography.

RESULTS

Expression of ets mRNA and Protein in Mouse Tissues. Products of protooncogenes have been implicated in events associated with cell growth and differentiation (24). To test whether the *ets* gene products are associated with germ-line differentiation—i.e., mitosis and meiosis—or with cell proliferation and differentiation, we have examined *ets* expression (both at RNA level and protein level) in wide varieties of young proliferating tissues and terminally differentiated adult tissues.

Poly(A)$^+$ RNA from young (5- to 10-day-old) and adult (5- to 6-week-old) tissues were analyzed by RNA blots using *ets-1*- and *ets-2*-specific probes (3). As shown in Fig. 1*B*, ets-1 mRNAs were detected as a major 5.3-kb and three minor 4.0-, 2.5-, and 2.0-kb species. Except for the thymus, the levels of ets-1 mRNA in young tissues were significantly higher in young tissue than in adult tissue. In general, the levels were ≈10 times lower in nonhematopoietic tissues such as testes, kidney, and heart than in thymus and young lung tissue. We could not detect *ets-1* expression in young brain and liver using the same amount of RNA as in other samples. The *ets-1* gene expression is greatly reduced in adult tissues, except for thymus. At this point, it is hard to assess whether this reduced expression is due to a higher turnover rate of ets mRNA or to a lower level of ets transcription. As shown in Fig. 1*A*, the rRNA level is approximately the same in all samples examined. Thus, it appears that the *ets-1* gene is expressed at high levels in tissues of hematopoietic origin.

On the other hand, the *ets-2* gene is transcribed as a major mRNA of 3.5 kb in a wide variety of tissues (Fig. 1*C*). These results were confirmed at the protein level, where 56- and 30-kDa ets-2 proteins were generally present at higher levels in young vs. adult tissues (Fig. 2). The level of these two proteins correlated very well with the ets-2 mRNA expression in young and adult thymus. The thymus is the only adult tissue examined that has both ets-2 RNA and protein expressed at a high level when compared to nonhematopoietic tissue. Unlike the 56-kDa ets-2 protein, the 30-kDa ets-2 protein is not detected by immunoprecipitation, suggesting that this 30-kDa protein may represent some cross-reacting species.

The ets-2 Expression During Liver Regeneration. Following partial (removal of two-thirds of the liver) hepatectomy, liver cells are stimulated to divide and the organ mass and cellular function is restored within 5–6 days (25). This model of cell proliferation provides an opportunity to study *ets* gene expression during pre- and post-replicative stages of the cell cycle, as well as the humoral factors regulating these events in normal, nontumorigenic tissue *in vivo*.

The kinetics of ets-2 mRNA induction was studied in preparations of poly(A)$^+$-selected RNA obtained from mouse

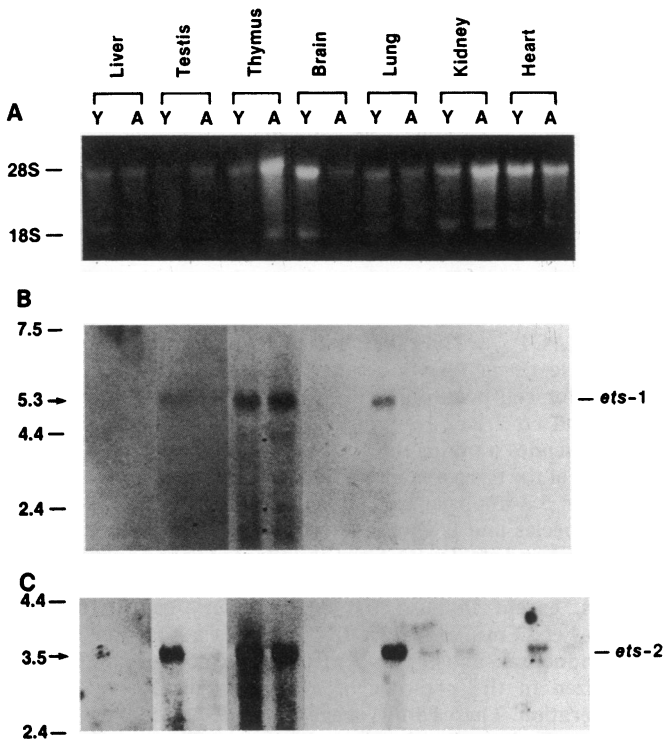

FIG. 1. Distribution of ets mRNA in young (Y) and adult (A) tissues. (*A*) Ethidium bromide-stained pattern of the residual rRNA. Three micrograms of poly(A)$^+$ RNA was fractionated on denaturing agarose minigel. After electrophoresis, fractionated RNAs were stained with ethidium bromide. (*B* and *C*) RNA blot analysis of ets mRNA. Twenty micrograms of poly(A)$^+$ RNA from young (5- to 10-day old) and adult (5- to 6-week old) tissues were fractionated and RNA blots were probed with human *ets-1*-specific probe (*B*) or with mouse *ets-2* probe (*C*) as described. The *ets-1* and *ets-2* transcripts are shown by arrows. Minor transcripts are indicated by dots. Bethesda Research Laboratories RNA ladder was used and standard RNA markers and their sizes (kb) are shown on the left.

livers at various times following partial hepatectomy. RNA blot analysis (Fig. 3) revealed that ets-2-specific mRNA expression peaks 4 hr after surgery and returns to basal level within 24 hr. Densitometric scanning of the RNA blots of ets-2 mRNA (data not shown), after partial hepatectomy, shows a 10-fold increase in the level of ets-2-specific mRNA at 4 hr in comparison to that observed in sham control and adult livers. These are minimal values, since the entire liver was used to prepare RNA rather than only the specific regenerated section. Clearly, the induction of ets-2 mRNA is due to the partial hepatectomy and not the stress induced by surgery, since only basal levels of ets-2 mRNA can be detected in sham control animals (Fig. 3). The amount of RNA applied in each lane is the same, as indicated by the nearly equivalent levels of rRNA present in all samples (Fig. 3). We have studied the DNA synthesis during the course of liver regeneration by the incorporation of [^{3}H]thymidine into DNA, and we found synthesis to peak at 48 hr (data not shown), consistent with published reports (25, 26).

We have also studied *ets* expression in quiescent BALB/c 3T3 fibroblasts following serum stimulation; both ets-1 and ets-2 RNA are increased 3 hr after serum addition, while DNA synthesis peaked at 16 hr (unpublished data). Thus, the maximal expression of ets-2 mRNA occurs well before DNA synthesis. The ets-1 mRNA was not detected following partial hepatectomy (data not shown) even at 4 hr, when the ets-2 mRNA level reached its maximum. This suggests that *ets-1* and *ets-2* loci are differentially regulated during hepatic regeneration.

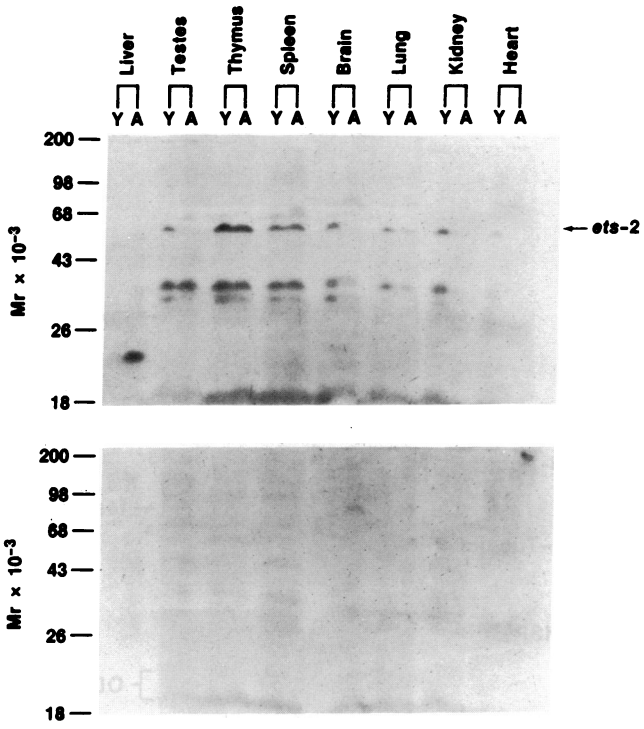

FIG. 2. Immunoblot analysis of ets-2 protein in young (Y) and adult (A) tissues. Two milligrams (wet weight) of tissue was solubilized in sample buffer and size-fractionated on $NaDodSO_4$/10% polyacrylamide gels. Immunoblots were processed in the absence (*Upper*) or in the presence (*Lower*) of cognate peptide as described. The position of the 56-kDa putative ets-2 protein is indicated by the arrow. Standard molecular weight markers are shown on the left.

Sequential Gene Expression During Liver Regeneration. To order the induction of ets-2 mRNA relative to the expression of other genes known to be involved in cell proliferation, we have analyzed mRNA from regenerating liver belonging to protooncogenes, *myc* and *fos* of the nuclear family, *mht* and *erbB* of the protein kinase family, and *sis* of the growth-factor family. In addition, we analyzed the expression of several nononcogenes, including genes for the structural proteins actin and tubulin, as well as the heat shock and metallothionein proteins. From densitometric scanning of RNA blots (Figs. 3 and 4), ets-2 mRNA levels were increased 10-fold in 4 hr and returned to basal level by 24 hr, and the pattern of myc mRNA accumulation was quantitatively different from that of ets-2. The myc mRNA levels increased 3- to 4-fold after 2 hr and returned to basal level by 8 hr (cf. Fig. 3); there was only a 2-fold increase in Ha-ras mRNA, which remained elevated up to 48 hr (Fig. 4). We were unable to observe any *fos-*, *mht-*, *erbB-*, or *sis*-specific transcripts during hepatic regeneration over the time points examined, but from RNA blot scanning data the actin mRNA was seen to increase 10-fold 4 hr after partial hepatectomy, and α-tubulin mRNA increased only 2- to 4-fold at 48 hr. Interestingly, expression of the metallothionein gene was markedly stimulated by the sham operation, whereas the heat shock protein gene was only slightly affected. These data are in agreement with published reports (27–32).

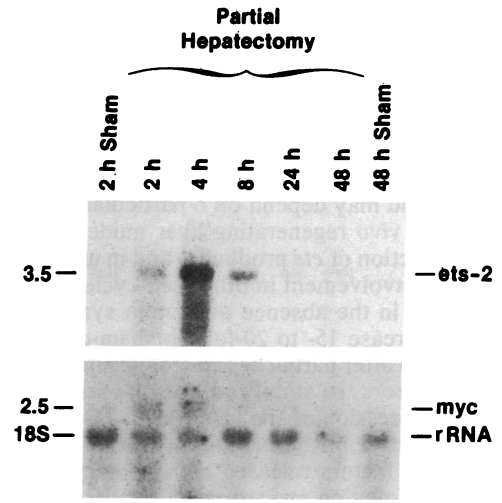

FIG. 3. Ets-2 gene expression in regenerating liver. Fifteen micrograms of poly(A)$^+$ RNA from sham control and livers from partially hepatectomized animals were fractionated on agarose gels and the filter was sequentially probed with mouse *ets-2* probe and human *MYC* probe. The transcript(s) detected by probes are indicated on the right and the size (kb) is shown on the left.

Superinduction of ets-2 mRNA During Hepatic Regeneration. The accumulation of mRNA in the presence of protein synthesis inhibitor has been termed as superinduction (33, 34). To test whether induction of ets-2 mRNA is dependent on new protein synthesis or not, we have studied *ets-2* gene expression (Fig. 5) in the presence of protein synthesis inhibitor (cycloheximide) before (upper left panel; protocols 1, 2, and 3) and after (*Upper left*; protocols 4, 5, and 6) partial hepatectomy. Like other nuclear protooncogene mRNAs (33, 34), cycloheximide alone increases ets-2 RNA by >15-fold without partial hepatectomy (Fig. 5, lanes 1 vs. 4 and lanes 4 vs. 5). In contrast, only low levels of ets-2 RNA were detected in sham-operated animals injected with either one or two doses of saline (Fig. 5, lanes 1 and 4). Partial hepatectomy amplifies (another 2- to 4-fold as determined by densitometry) the effect of cycloheximide on *ets-2* expression (Fig. 5, lanes 3 vs. 2 and lanes 6 vs. 5); this superinduction effect was not observed for the *ets-1* gene, which was not expressed even after such procedures. *myc*, *fos*, and ornithine decarboxylase (a marker for cell proliferation) share expression patterns similar to that of the *ets-2* gene.

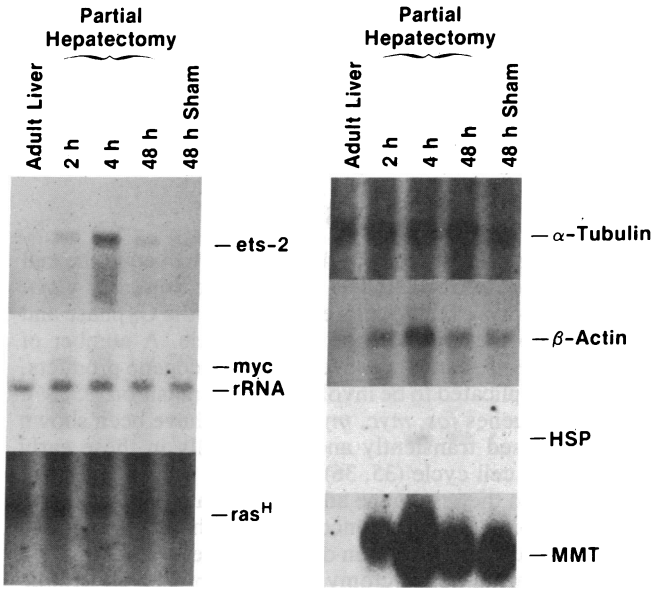

FIG. 4. Gene expression during liver regeneration. Fifteen micrograms of poly(A)$^+$ RNA from adult liver, sham control livers, and livers from partially hepatectomized animals were size-fractionated and filters were probed sequentially with mouse *ets-2*, human *MYC*, v-Ha-*ras* (ras^H), α-tubulin, β-actin, heat shock protein (HSP), and mouse metallothionein (MMT) probes. Transcripts detected by these probes are indicated on the right.

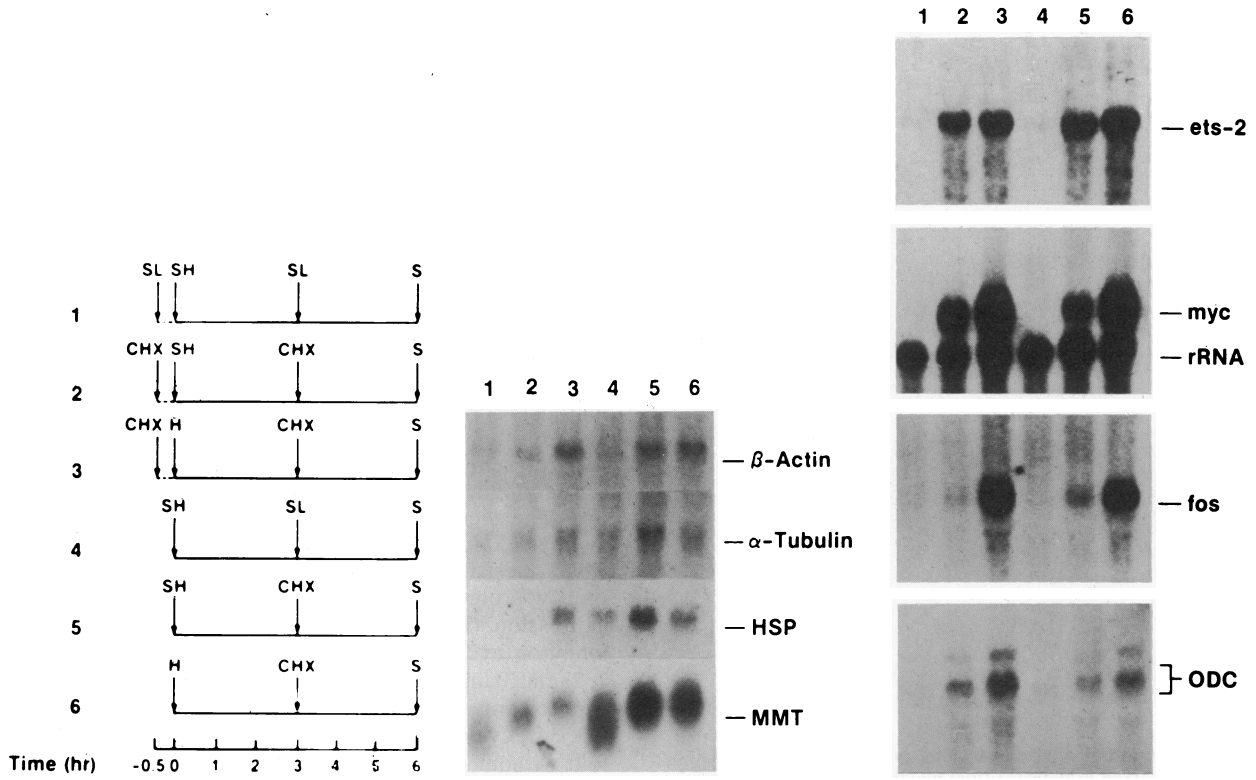

FIG. 5. Superinduction of ets-2 mRNA during liver regeneration. (*Left*) Protocol used to superinduce ets-2 mRNA. Either 0.15 ml of saline (SL) or 0.15 ml of cycloheximide (CHX) in saline (100 mg per kg of body weight) was injected intraperatorially at the indicated time. SH, sham operation; H, partial hepatectomy; S, sacrifice. (*Center* and *Right*) Fifteen micrograms of poly(A)$^+$ RNA from each sample (as described in *Left*) was size-fractionated and filters were probed sequentially with mouse *ets-2*, human *MYC*, human *FOS*, and mouse ornithine decarboxylase (ODC), β-actin, α-tubulin, heat shock protein, and metallothionein probes. Transcripts detected by these probes are indicated on the right.

These data further establish that *de novo* protein synthesis is not necessary for the induction of *ets-2, fos, myc*, and ornithine decarboxylase genes. However, superinduction—i.e., the synergistic effect of cycloheximide and partial hepatectomy—is not observed for β-actin, α-tubulin, heat shock protein, and metallothionein protein genes (Fig. 5). Thus, stabilization of ets-2 mRNA after addition of cycloheximide allows us to identify *ets-2*, being part of the proliferative gene family and demonstrates further that, during hepatic regeneration, both *ets-1* and *ets-2* genes are differentially regulated.

DISCUSSION

Most of the information about the events involved in the cell cycle have come from studies with cells cultured *in vitro*. Initially, density-arrested cells emerge from the G_0 phase and progress into the G_1 phase of the cell cycle. A number of specific proteins, including several protooncogene products, have been implicated to be involved in this transition (35, 36). The nuclear genes *fos, myc, myb*, and p53 have been shown to be expressed transiently and sequentially in these early stages of the cell cycle (35, 36).

Our data on the *ets-2* gene add to this scant listing another transiently expressed protooncogene, which may be required for the proliferative transition of hepatocytes following stimulation by partial hepatectomy. The data presented demonstrate that partial hepatectomy induces the expression of ets-2 mRNA, which reaches a peak by 4 hr and returns to basal levels by 24 hr, while DNA synthesis occurs much later, around 45–48 hr. This observation indicates that the *ets-2* gene is effectively transcribed well before DNA synthesis, thus implicating this gene product in events necessary and prior to cell division.

Consistent with the RNA data, antibodies prepared against an *ets-2*-specific peptide identify a 56- and 30-kDa protein in young proliferating tissues (Fig. 2) as well as in some adult tissues. The 30-kDa polypeptide may represent degradation product of the 56-kDa protein. However, we have tentatively identified the 56-kDa polypeptide as being a translation product of ets-2 mRNA (unpublished data); we have also found from preliminary subcellular localization studies using mouse tissues and human cell lines that this product of the *ets-2* gene is located in the nucleus. Taken together, these data implicate the *ets-2* gene as a member of the family of nuclear protooncogenes. The nuclear protooncogenes, expressed normally during the early phase of the cell cycle, appear to have another property in common; their RNAs are stabilized in the presence of cycloheximide (33, 34). Although the properties affecting stabilization may vary from cell line to cell line (34) and may depend on a particular stage of the cell cycle, the *in vivo* regenerating liver model is useful in studying the induction of *ets* product(s) and in understanding the nature of its involvement in other cell-cycle events. Our observation that, in the absence of protein synthesis, ets-2 mRNA levels increase 15- to 20-fold in sham-operated animals and ≈40-fold after partial hepatectomy (Fig. 5) suggests that inducible factors, so stabilized, may be responsible for the increased expression of the *ets-2* gene. In addition, the higher levels of ets-2 mRNA could be due to the additive effects of growth factors released after surgery and subsequent signal-transduction processes.

Several observations on cellular proliferation using *in vitro* systems have contributed evidence substantiating the mitogenic activation of specific protooncogenes in an orderly fashion during the early stage of cell division (35, 36). The activation of *ets-2* transcription can be assigned following the induction of the other nuclear protooncogenes, *fos* and *myc*,

in an *in vivo* liver regenerating system (28–32). Thus, the early phase of hepatic regeneration can be characterized by the following sequential expression of cellular protooncogenes: *fos*→→*myc*→→*ets-2*. We do not know if these genes are obligatorily expressed in this unique sequence, or if the gene transcripts are processed at different rates and thus appear so ordered. We do know, however, that during hepatic regeneration the transcription of the *ets-2* protooncogene does not require *de novo* protein synthesis.

Tissues that contain actively dividing cells would be expected to have high levels of expression of those genes involved in cell proliferation, whereas terminally differentiated tissues would not. Consistent with this idea, we have found lower levels of expression of ets-2 RNA and proteins in adult liver, brain, kidney, lung, heart, and testes than in young proliferative tissues (Figs. 1 and 2), but this appears not to be the case in thymus tissue. Since we found no evidence of gene rearrangement of the *ets* loci in young and adult testes (data not shown), we can suggest other mechanisms, such as DNA methylation, lower rates of gene transcription, or higher turnover rates of mRNA, as being responsible for the lower levels of ets-2 mRNA observed in adult tissues. Thymus, a highly proliferative tissue, appears to have the highest level of *ets* gene expression; indeed both ets-1 and ets-2 mRNA do not vary much over the course of its development (Fig. 1). This high level of *ets* expression could be due to hormonal and/or physiological microenvironment involved with T-cell maturation. Interestingly, the expression of another nuclear protooncogene, *myb* (also part of the E26 virus) is also seen not to vary significantly with the age of the thymus (37).

We have shown previously that both *ets-1* and *ets-2* protooncogenes are located on different chromosomes in Mammalia and each locus is transcriptionally active (3, 4). In this paper, our finding that only ets-2 mRNA is expressed in the early phase of hepatic regeneration demonstrates that these two distinct protooncogenes are differentially regulated in liver. This hypothesis is further supported by our observation that ets-1 mRNA is not superinduced.

In summary, our results suggest that in liver both *ets-1* and *ets-2* gene expression are differentially regulated; *ets-2* gene transcription is induced by hepatectomy; and expression of the *ets-2* gene product is similar to the pattern of expression for the *fos* and *myc* nuclear genes. Therefore, it logically follows that the *ets-2* gene product is associated with the cell cycle. This involvement is in keeping with the role postulated for the other nuclear protooncogenes.

We would like to thank our colleagues for providing us with the probes used in this study, especially Drs. D. K. Watson for *ets*, Nancy Kan for *mht*, J. A. Lautenberger for v-Ha-*ras* and v-*sis*, and Ken Samuel for v-*myb*. We would also like to thank Drs. Morimoto for heat shock protein, F. Berger for ornithine decarboxylase, G. N. Pavlakis for metallothionein, Marianne Oskarsson for c-*mos*, Donald Cleveland for α-tubulin, and S. Farmer for β-actin. Special thanks to Karen Cannon for expert preparation of this manuscript.

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