The *ets* Genes in Cells and Viruses: Implications for Leukemias and Other Human Diseases

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One of the most interesting scientific challenges of our time has been to understand the spectrum of oncogenic changes necessary to commit normal cells to a neoplastic life-style. On the molecular level, this enigmatic quest centers upon our understanding the essential ingredients needed to control cell growth (and its coordinate processes), as well as to maintain the normal differentiated pattern of expression for specific genes. Another, perhaps simpler, approach to this difficult challenge would be to examine the genetic changes resulting from the introduction of a known acute retroviral transforming sequence capable of eliciting a malignant cellular phenotype *in vitro* and neoplastic disease *in vivo*. Through the application of the powerful technique of recombinant technology application in the study of model retrovirus systems, the precise DNA sequences responsible for cellular transformation have been identified and studied. Thus, the employment of acute retroviruses as tools to identify a select cohort of genes capable of malignant transformation has been most fruitful. More important, from this study came a realization that this category of genes, the viral oncogenes, were capable of establishing and maintaining the transformed state, as well as the recognition that these genes were derivatives of a limited population of normal cellular genes, the proto-oncogenes, that can be captured and modified by the viral transduction process. These homologous genes, now numbering several dozen, are found distributed in the genomes of almost all vertebrates and of some invertebrate species, as well as of a few more primitive single-cell species.

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Some general conclusions have been evolving from the study of these viral oncogenes and their normal cellular homologs, namely, that the transforming variants are damaged, frequently truncated versions of the normal cellular proto-oncogenes.[1] Thus, this avenue of study provides scientists and oncologists with some direction and serves to focus research at many levels. These investigations, be they biochemical, genetic or clinical have pointed to common cellular denominators implicating these oncogenes and proto-oncogenes and their products in certain critical processes in which they may be involved.[2] Added to these investigations is the cumulative evidence that these oncogene homologs are located at critical sites on human chromosomes. These loci may be subject to nonrandom chromosomal aberrations and thus be involved in quantitative or qualitative changes associated with human neoplasia and other diseases.[3]

Studies in our laboratory have centered on the use of retroviruses as tools to investigate avian acute leukemia virus transforming genes and their normal cellular counterparts. As a result of these studies, we have come to realize that specific viral oncogenes like those belonging to the avian erythroblastosis virus, E26, not only contribute significantly to a growing body of information related to that class of conserved cellular genes that may be important in tumorigenesis, but may also be functionally important in cell growth and, as such, relevant to other human diseases.[4]

The transforming sequences of the avian acute leukemia virus, E26, contain two distinct oncogenes, v-myb^E and v-ets, fused together with segments of the viral structural genes, Δ-gag and Δ-env, to constitute the 5.7-kilobase (kb) genome of this replication-defective retrovirus[5,6] (FIG. 1).

The E26 virus induces both myeloblastic disease and erythroblastosis *in vivo*. The myeloid oncogenic property that the E26 virus and avian myeloblastosis virus (AMV) share in common is thought to be due to their common transduction of *myb* coding domains. The erythroblastosis pathology of E26 virus, on the other hand, may be due to the presence of the unique v-*ets* domain or perhaps to a synergistic effect of both oncogenes. The nucleotide sequence of a 2.5-kb *ets*-containing region of E26 DNA reveals a contiguous *gag-myb-ets* open reading frame encoding a 135-Kd protein, p135, a product found in E26 viral-transformed cells.[5,7]

To study the genetic organization and origin of the *ets* oncogene, our laboratory has cloned and studied the molecular details of the proto-*ets* gene originating from the avian host (*i.e.*, the chicken), as well as compared these proto-*ets* genes in a variety of other species of animals, including man.[8] To facilitate our investigations on the comparison of chicken proto-*ets* genes, with the viral *ets* genes, overlapping clones were isolated and digested with different restriction enzymes and then hybridized with specific segments of v-*ets* probes and aligned. Nine presumptive exons were thus defined by their homology to v-*ets*, each exon delineated by consensus splice-acceptor and splice-donor sequences. These cellular exons ranged in size from 82 to 257 base pairs, while the introns are seen to be quite variable, ranging from 0.1 kb to greater than 40 kb. The v-*ets* homologous chicken sequences are seen to be spread over 60 kb of genomic DNA, making it one of the most dispersed proto-*onc* genes characterized thus far (FIG. 2). As expected, since the chicken is the retroviral host organism, there is a very high conservation of the majority of *ets* sequences found in the virus; all but *three* nucleotides are unchanged from chicken to virus. Moreover, the chicken and the viral genes are not coterminal.[9] However, this observation is consistent with our data obtained from human genomic and cDNA clones demonstrating that the human *ets*-2 and v-*ets* genes are not coterminal.[10]

Using segments of the v-*ets* oncogene as probes, we have been able to identify proto-*ets* sequences in the human genome, as well as in the genome of other mammals (feline and murine). In humans there are two distinct v-*ets* homologous loci, Hu-*ets*-1

and Hu-*ets*-2. These human loci were found to be discontiguous unlike that of chicken and avian E26 virus except for a small region of overlap.[10] On the basis of structural organization and molecular sequence comparisons, we have defined the 5' portion of the viral *ets* oncogene as *ets*-1, and the 3' portion of the viral *ets* oncogene as *ets*-2. These proto-*ets* domains in mammals map to separate loci on homologous, but distinctly different chromosomes. Thus, the 5' v-*ets* homologous region defined above locates in man (*i.e.*, human proto-*ets*-1 gene [Hu-*ets*-1]) on chromosome 11; its feline and murine counterparts locate on chromosomes (f)D1 and (m)9, respectively. The 3' v-*ets* homologous region similarly locates in man (*i.e.*, human proto-*ets*-2 gene [Hu-*ets*-2]) on chromosome 21, while the feline and murine homologs are on chromosome (f)C2 and (m)16, respectively. Each of the *ets* genes characteristically belong to homologous syntenic groups in *Mammalia* with respect to other genetic markers (FIG. 3).[11]

Scrutiny of the nucleotide sequences of the cloned human proto-*ets* sequences with their viral *ets* homolog reveals a striking level of conservation. The Hu-*ets*-1 and Hu-*ets*-2 genes, respectively, possess a 98% and 95% homology with their viral counterparts, comparing at the amino acid level. This homology would place these genes among those highly conserved between cellular and viral oncogene sequences. Most surprising, however, has been our observations on the cloned *ets*-2 homologous sequences in *Drosophila*; this homolog, containing only the last two exons of *ets*, shows over a 90% conservation at the amino acid level compared with the Hu-*ets*-2 gene—a
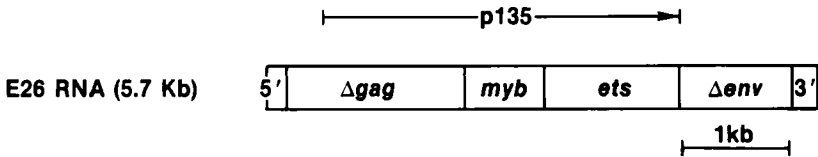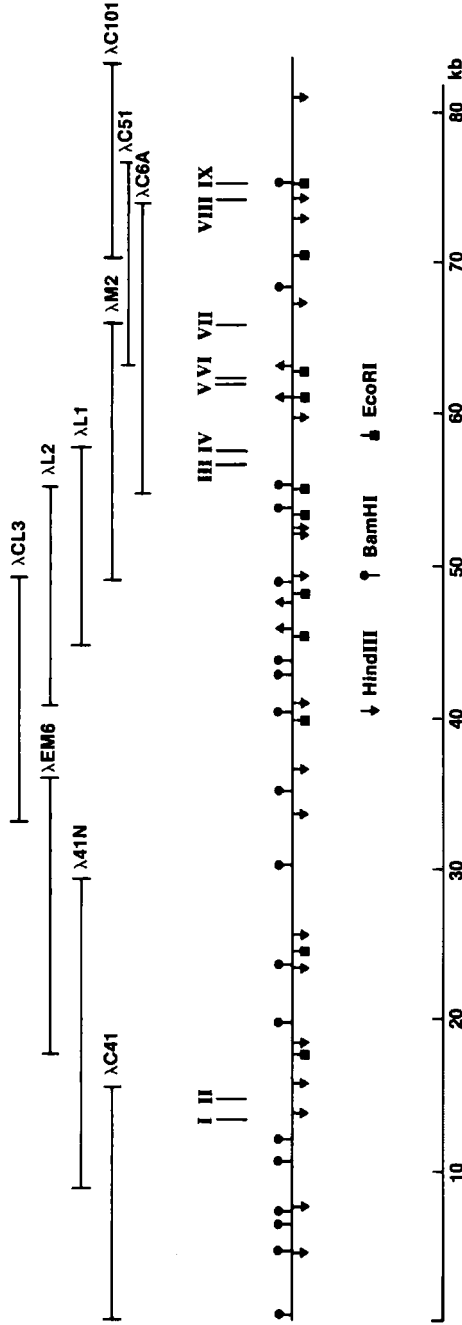

FIGURE 1. Genomic organization of the avian acute transforming virus, E26.

remarkable level of conservation; also the highest thus far noted for any proto-oncogenes so widely separated on the evolutionary scale (FIG. 4).[12]

Our laboratory has been able to determine that both the human *ets*-1 and *ets*-2 loci are transcriptionally active and are differentially expressed; the Hu-*ets*-1 gene is transcribed as a single 6.8-kb mRNA, while the Hu-*ets*-2 gene is expressed as three distinct species of RNA, sized 4.7, 3.2 and 2.7 kb. By contrast, the chicken c-*ets* mRNA is five times more complex (7.5 kb) than the viral *ets* genomic RNA (1.5 kb).

Interestingly, the viral *ets* contains in its 5' domain, the v-*ets*-1 region, 224 nucleotides not found transcribed in chicken thymus. Thus, not only has the E26 virus captured a much smaller portion of the chicken *ets* gene, but it has also transduced a substantial portion of cellular noncoding sequences that are now able to be incorporated into the E26 viral-encoded product.[13] These molecular events, in addition to the carboxy terminal differences noted above, probably have important functional implications for the retroviral transforming protein, as well as its biochemical role in cellular transformation.

As stated above, the products of the mammalian *ets*-1 and *ets*-2 genes appear to be differentially expressed[14] and we shall present evidence later in this report that their encoded protein products are localized predominantly in separate subcellular compartments.[15] Specifically, the human *ets*-1 gene-encoded protein seems to locate preferentially in the cytoplasm, while the human *ets*-2 gene-encoded protein appears in the

FIGURE 2. Genomic organization of the contiguous chicken *ets-1/ets-2* gene. Diagrammed on top are the endpoints of the genomic DNA inserts contained in 10 overlapping λ clones as determined by restriction enzyme analysis and hybridization. The positions of the 9 viral homologous domains are illustrated and denoted by roman numerals above the restriction map of the chicken proto-*ets* locus.

nucleus. It is known that a number of proto-oncogene products, particularly of the nuclear family, have been implicated in cellular systems as regulators and effectors of proliferation.

To investigate the possibility that the *ets* genes may be involved in such growth-related processes we studied its expression in a variety of murine tissue, including germ-line tissue (testis). The expression of *ets*-1 was much higher in cells and tissue of hematopoietic origin than in other tissue, including hepatic tissue. The murine expression of *ets*-1, as characterized by multiple species of mRNA, 5.3, 4.0, 2.5 and 2.0 kb in size, was found to be actively transcribed in both young and old thymus tissue. By contrast, the product of the murine *ets*-2 gene, characterized by a 3.5-kb transcript, was preferentially expressed in a variety of proliferating young tissue by contrast to less proliferative older tissue. This relative difference was most clearly evident in testis tissue, where *ets*-2 mRNA synthesis was maximal in 8-day-old preparations, compared to older (42-day) germ-line-derived samples (FIG. 5). This observation suggested that the proliferative state of the tissue may be relevant to the expression of *ets*-2. In fact, for these age groups, even for the exception noted, the abundant *ets*-2 expression in thymus tissue tended to prove the rule, since thymocytes are normally in a highly proliferative state. By contrast, there was significant lower expression of *ets*-2 in terminally differentiated adult brain, liver, kidney, lung and heart tissue, which were compared with appropriate controls, using an α-tubulin gene probe. Thus, it appeared from these data that the *ets* genes are differentially expressed and subject to variable regulation in proliferating tissue (FIG. 5).[14,16] Studies on the expression of *ets*-1 and *ets*-2 gene-encoded protein also substantiated these observations (FIG. 6). Antisera raised against an *ets*-2 domain specific oligopeptides and bacterially-expressed *ets* proteins detected two *ets* gene proteins (56 KDa and 30 KDa), which appeared to correlate very well with the *ets*-2 mRNA expression observed in young and adult thymus. The thymus is the only adult tissue examined that had both *ets*-2 RNA and protein expressed at a high level when compared to nonhematopoietic tissue. Unlike the 56-KDa *ets*-2 protein, there was a selected species not detected by immunoprecipitation, suggesting that this 30-Kd protein may represent some cross-reacting species.

To further elucidate the linkage of *ets*-2 gene expression with cellular proliferation, we studied hepatically regenerating tissue as a model *in-vivo* system. It is known that when two thirds of the liver mass is removed by surgery in the mouse,[17] the remaining liver cells proliferate and organ mass and function is completely restored within 5 to 6 days posthepatectomy.[18] FIGURE 7 depicts a result of our Northern transfer analysis of poly A-selected RNA obtained at various times postpartial hepatectomy. Data shows clearly that the *ets*-2 mRNA peaks 4 hrs following surgery, and returns to basal levels within 24 hrs. Densitometric scanning studies of the 4-hr sample of *ets*-2 mRNA, following partial hepatectomy, shows a 10-fold increase above basal levels found in sham controls or in adult liver tissue. It should be kept in mind that these are minimal values, since the entire liver was used to prepare RNA rather than the specific regenerated tissue. 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 samples. The amount of RNA applied in each lane was the same, since there were equivalent levels of rRNA present in all samples tested.

During the course of liver regeneration DNA synthesis peaked at 48 hrs following surgery, consistent with other published reports;[17] thus, the maximal expression of *ets*-2 occurring at 4 hrs after hepatectomy is well before that of DNA synthesis. Hepatic *ets*-1 expression, unlike that of *ets*-2, was not detected following partial hepatectomy at any of several time points spanning one round of DNA replication, further corroborating that these genes are differentially expressed.
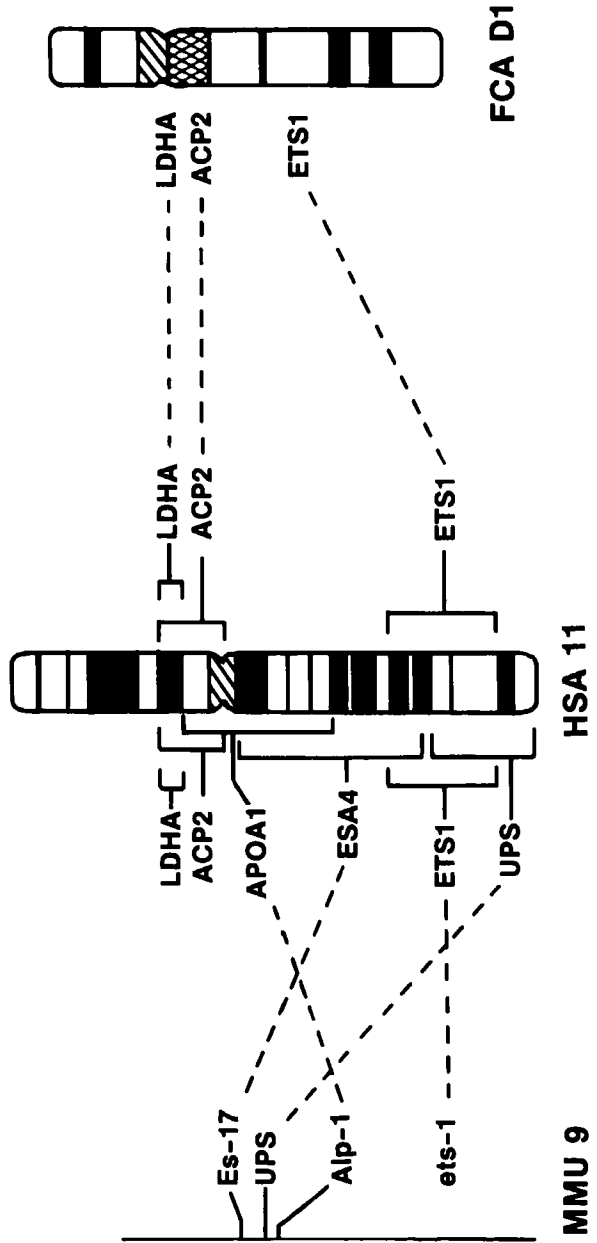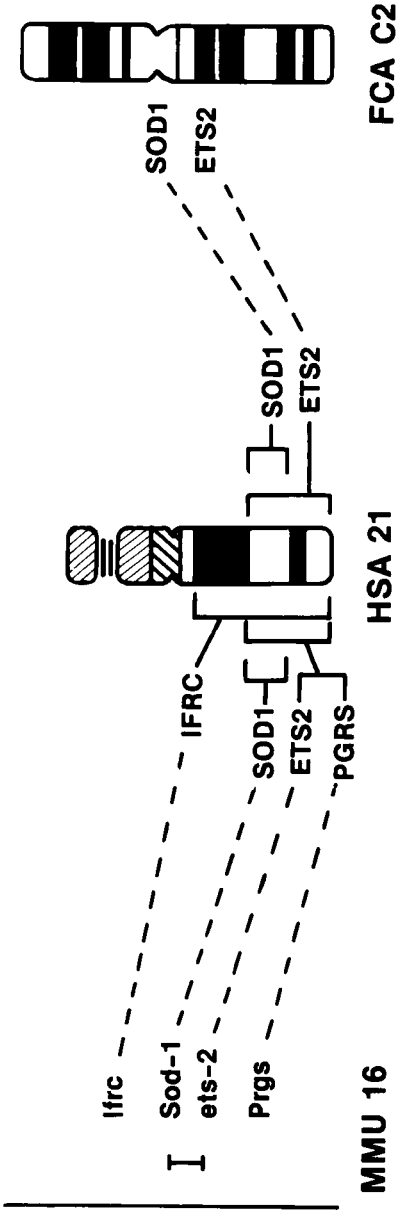
FIGURE 3. Diagram of regional positions of homologous loci on chromosomes to which *ets*-1 and *ets*-2 have been assigned in man (HSA), mouse (MMU), and cat (FCA). Basis for homology of included genes is discussed in Roderick *et al.* (1984), and O'Brein, Seuanez, and Womack (1985).
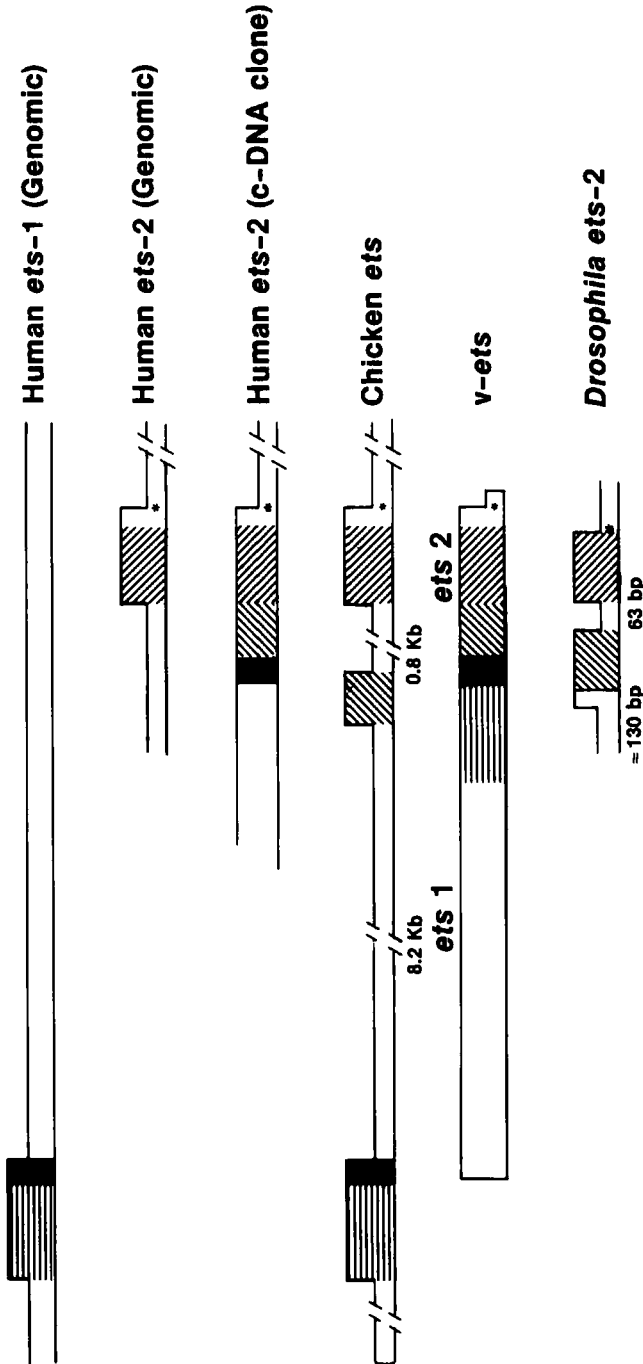
FIGURE 4. Alignment of the *ets* sequences and exons. In this diagram the human *ets*-1 genomic and Hu-*ets*-2 genomic and cDNA clones are aligned and compared with the chicken *ets* genomic clone; the v-*ets* clone and the *Drosophila ets*-2 clone. The *horizontal hatching* depicts the last exon of the *ets*-1 region; the coding segments are indicated by *full-height*, and the noncoding segments by *half-height unshaded regions*, and the last two *ets*-2 exon domains are depicted by the *slanted (up and down angled) hatching*, respectively. The *solid black region* indicates the common 14 amino acid overlaps between the *ets*-1 and *ets*-2 domains; the *asterisk* (*) signifies the sequence termination sites.
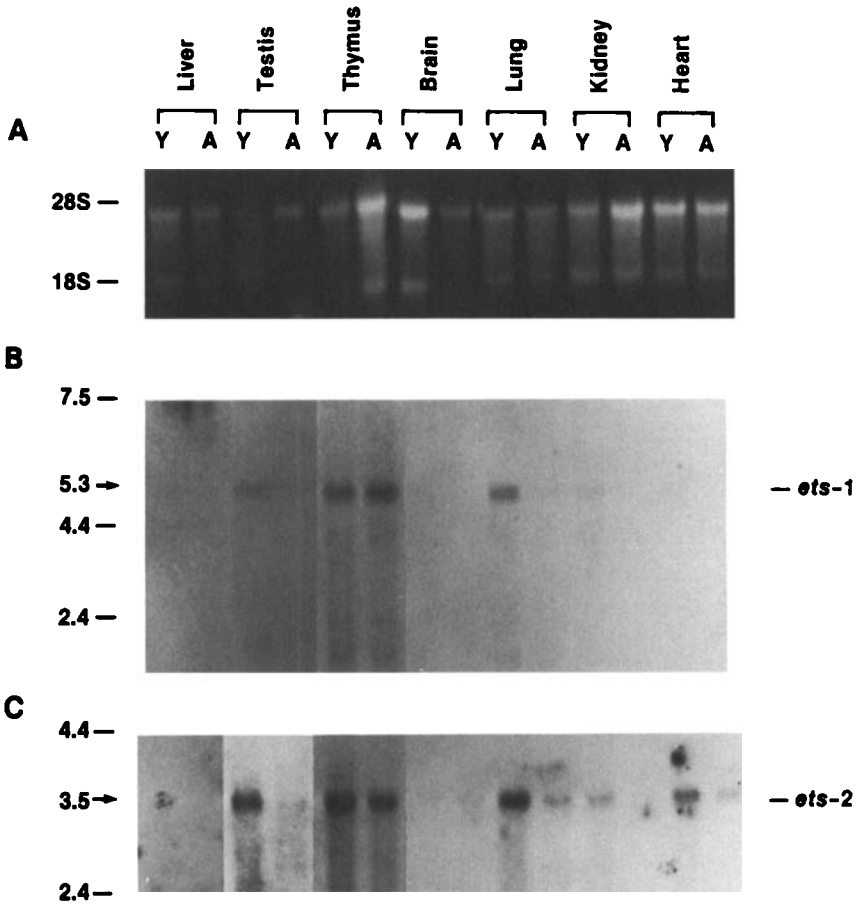
FIGURE 5. Distribution of *ets* mRNA in *Young* (Y) and *Adult* (A) tissues; (A) Ethidium bromide stained pattern of the residual rRNA. Three micrograms of poly A$^+$ RNA was fractionated on denaturing agarose min-gel after the electrophoresis fractionated RNAs were stained with ethidium bromide. (**B and** C) Northern 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 Northern blots were probed with human *ets*-1-specific probe (B) or with mouse *ets*-2 probe (C) as described in the text. The *ets*-1 and *ets*-2 transcripts are shown by *arrows*. Minor transcripts are indicated by *dashes*. BRL RNA ladder was used as standard RNA markers and their sizes (kb) are shown on the *left hand side*.

To arrange the induction of *ets*-2 mRNA in an ordered sequence of expression relative to other genes known to be involved in cell proliferation, we examined the kinetics of expression of several select genes. Specifically, we probed several proto-oncogenes: *myc* and *fos* of the nuclear family; *mht*, *erb*B of the protein kinase family; and *sis* of the growth factor family. Also, we selected several non-oncogenes representing the housekeeping family, actin, tubulin, and those of a stress-related family, the heat shock protein, and a metallothionein protein. The *ets*-2 mRNA levels increased 10-fold in 4 hrs, returning to basal levels by 24 hrs; whereas the kinetics of
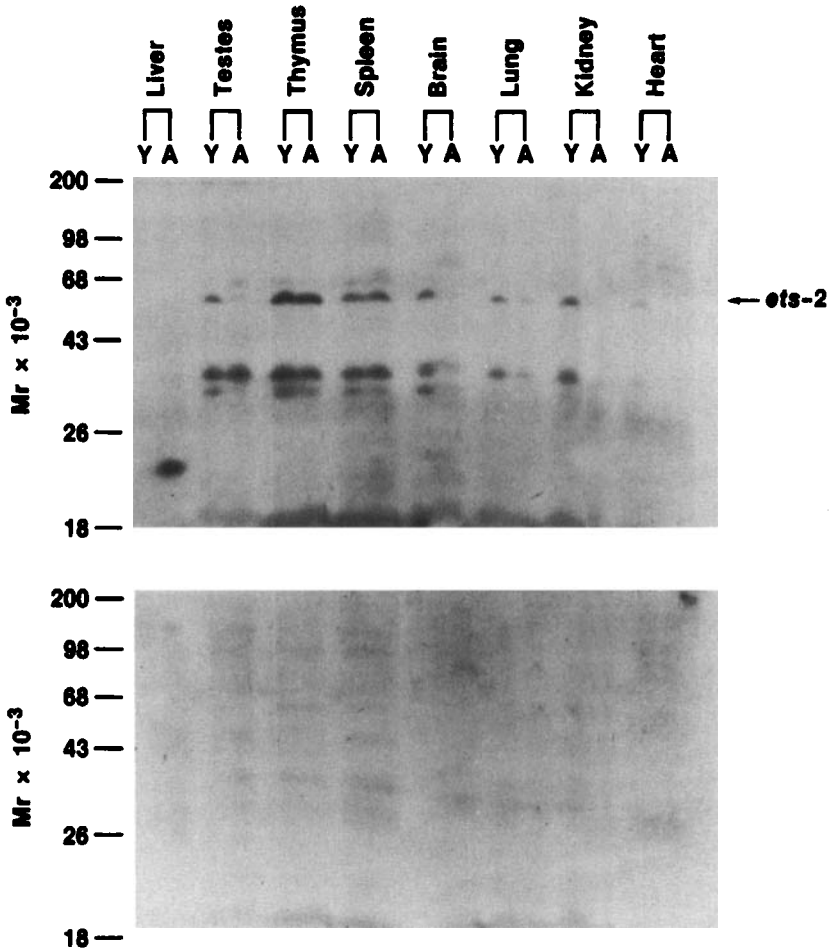
FIGURE 6. Western blot analysis of *ets*-2 protein in *Young* (Y) and *Adult* (A) tissues. Two milligrams (wet/wt) tissues were solubilized in sample buffer and size fractionated on 10% polyacrylamide SDS gels. Western blots were processed in the absence (*top panel*) or in the presence (*bottom panel*) of cognate peptide as described in the text. The position of the 56-Kd putative *ets*-2 protein is indicated by the *arrow*. Standard molecular weight markers are shown on the *left hand side*.

myc and *ras*[H] mRNA induction is notably different from that of *ets*-2. Specifically, the *myc* RNA level increased 3- to 4-fold after 2 hrs and returned to basal level by 8 hrs. By contrast, *ras*[H] mRNA increased 2-fold and remained elevated even after 48 hrs. The proto-oncogenes *fos*, *mht*, *erbB* or *sis*-specific transcripts were not detected at different time points examined during hepatic regeneration. Note, however, that the metallothionein messenger RNAs increased more than 100-fold in both sham-operated and hepatectomized animals, reflecting the stress imposed by surgery. Actin mRNA, too, was induced, but only 10-fold, 4 hrs following partial hepatectomy; this course of induction was different from the α-tubulin mRNA, which increased 2- to 4-fold after

48 hrs. Failure to detect expression of the proto-oncogene, c-*fos*, which is transiently expressed, was expected, since our earliest time point sampled was 2 hrs posthepatectomy; this is a time well past the known maxima of c-*fos* induction.[19] Collectively, these data are in agreement with the sequential expression of nuclear proto-oncogenes that are known to induce during the transition from G_0–G_1 phase of the cell cycle following partial hepatectomy.[20]

Like other nuclear proto-oncogene mRNAs, cycloheximide was found to superin-
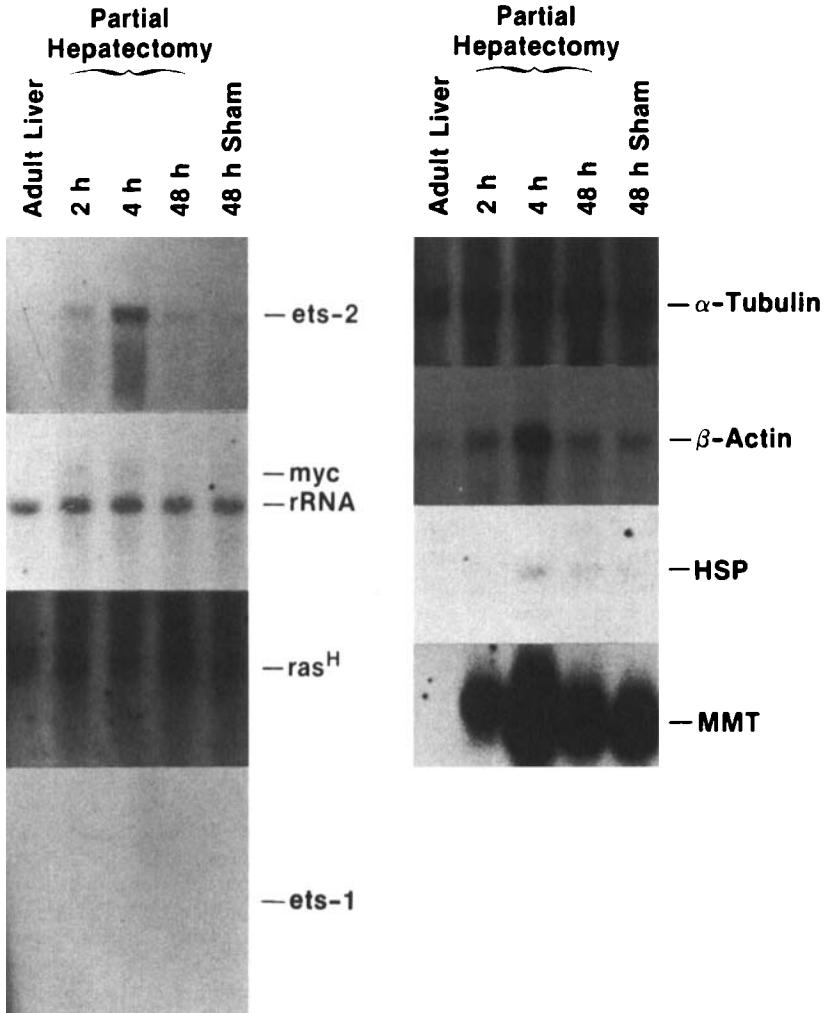
FIGURE 7. Gene expression during liver regeneration. Fifteen micrograms of poly A^+ RNA from adult liver, sham control livers, and livers from partially hepatectomized animals were size fractionated and filters were probed sequentially with mouse *ets*-2, human *ets*-1, human *myc*, v-*ras*^{H}, α-tubulin, β-actin, heat shock protein (HSP) and mouse metallothionein (MMT) probes. Transcripts detected by these probes are indicated on the *right hand side*.

duce the level of *ets*-2 RNA by more than 15-fold, as well as stabilizing the *ets*-2 message. Partial hepatectomy amplified (20- to 40-fold) the effect of this *ets*-2 expression, which was not observable for the *ets*-1 mRNA. *Myc*, *fos* and ornithine decarboxylase (a marker for cell proliferation) also shared the same expression pattern as the *ets*-2 gene. Collectively, these data help establish that the *ets*-2 gene is a member of a proliferative gene family, and one that is subject to differential regulation and expression in proliferating hepatic tissue. Our data implicate the *ets*-2 gene as a member of the family of nuclear proto-oncogenes that is normally expressed during the early phase of hepatic regeneration and can be characterized by the following sequential expression, *fos* →→→ *myc* →→→ *ets*-2 →→→. We do not know as yet if these genes are obligatorily expressed in this particular 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 proto-oncogene does not require *de-novo* protein synthesis.

To summarize, our results on the studies of *ets* RNA in mice suggest that in murine liver both *ets*-1 and *ets*-2 gene expression is differentially regulated; that the *ets*-2 gene transcription is inducible by hepatectomy and its expression is similar to the pattern of expression noted for other nuclear proto-oncogenes, *fos* and *myc*. It logically follows that the *ets*-2 gene product may be associated with the transition of cells from G_0 to G_1 phase of the cell cycle and this involvement is certainly consistent with the roles postulated for other nuclear proto-oncogenes.

We have recently presented information identifying the products of the two human *ets* genes and their subcellular location.[15,21] To accomplish this study, we developed antibodies specific for *ets*-1 and *ets*-2 domains. Another antibody was prepared using a synthetic oligopeptide derived from the predicted amino acid sequences of the *ets* common domain; a portion of this region has been highly conserved in a variety of species, ranging from *Drosophila* to man.[8,12,22] All of these antibodies are described in the legend of FIGURE 8.

We established that these three antibodies reacted specifically with *ets* proteins by immunoreactive studies using extracts of transfected NIH3T3 cells infected with a murine/avian recombinant retrovirus construct containing the *gag-myb-ets* region of the E26 virus. The [^{35}S]-methionine-labeled extracts of these cells were each immuno-precipitated using the three different antibodies. In all three experiments the antibodies precipitated the 135-KDa *ets*-containing fusion protein (FIG. 8, lanes 1, 3 and 5). Moreover, precipitation of this v-*ets*-encoded protein was specifically blocked by an excess amount of the respective peptide-antigen (FIG. 8, lanes 2 and 4). The p135 fusion protein was found only in transfected cell extracts and was also recognized by sera prepared against the avian retroviral *gag* protein. There is additional evidence, FIGURE 8, showing that these antibodies react with *ets*-specific sequences, since the *ets*-1-specific antibody also reacted with a v-*ets*-1 protein expressed in bacteria (FIG. 8, lane 6), and the *ets*-1-*ets*-2 common domain antibody detected a bacterially-expressed *ets*-2 protein product (FIG. 8, lane 8). Both reactions were immunospecific, since excess peptide totally competed the precipitation of the respective *ets* proteins (FIG. 8, lanes 7 and 9).

Having reported previously that *ets*-1 and *ets*-2 domains are contiguous in the avian genome,[10] we had to determine whether the *ets*-1 and *ets*-2 antibodies are able to detect a singular protein in chicken tissue, using for this purpose [^{35}S]-methionine-labeled chicken thymus cells known to express high levels of *ets*-mRNA.[14] Not surprisingly, antibodies against the *ets*-1 domain and *ets*-1-*ets*-2 common domain also immunoprecipitated chicken *ets*-1-*ets*-2-encoded protein (FIG. 8, lanes 10 and 12).

To identify and characterize the human proteins derived from the *ets*-1 and *ets*-2 loci, we have utilized cell lines expressing high levels of either *ets*-1 or *ets*-2 mRNA (N.

Bhat, unpublished observations).[14] For example, [^{35}S]-methionine-labeled Daudi cells, the Burkitt lymphoma cell line, was employed to detect the human *ets*-1 grotein, with our *ets*-1-specific antibody. As can be seen, a prominent 51-KDa protein was detected (FIG. 9, lane 1), which was also detected using the common *ets*-1-*ets*-2 domain antibody (FIG. 9, lane 3). However, the antibody derived from the *ets*-2 region does not precipitate the p51 *ets*-1 protein (FIG. 9, lane 5).
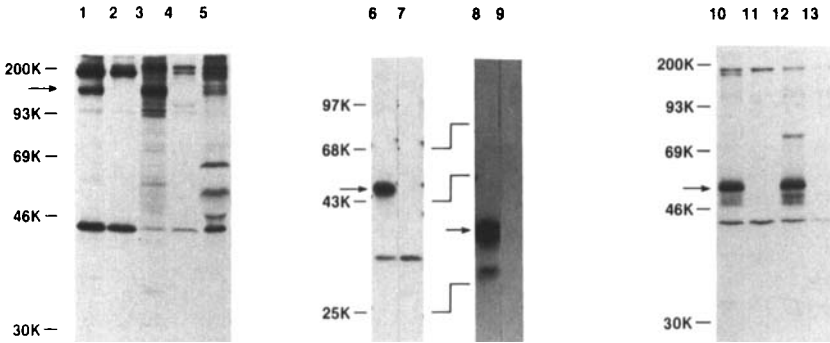
FIGURE 8. Specificity of the *ets* antibodies. Antibody A (*ets*-1-specific), Antibody B (*ets*-1-*ets*-2-specific) and Antibody C (*ets*-2-specific) have been described.[15,21] In *lanes 1 through 5,* the p135 *gag-myb-ets* protein was immunoprecipitated from extracts of NIH3T3 cells transfected with the murine/avian recombinant retrovirus construct (Yuan, Q., Blair, D., Papas, T., unpublished results) that were labeled with [^{35}S]methionine (100 μCi/ml, 1 h) and lysed using RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1.0% Triton X-100, 0.5% deoxycholate, 0.1 mM EGTA, 1.0 mM EDTA, 0.4 mM phenylmethyl-sulfonyl fluoride (PMSF), 0.01 mM Nα-p-Tosyl-L-lysine chloromethyl ketone (TLCK), 0.1 mM N-Tosyl-L-phenylalanine chloromethyl ketone (TPCK)). The lysate was clarified by ultracentrifugation (100,000 × g, 30 min) and subjected to immunoprecipitation by the rabbit antibodies prepared against the synthetic oligopeptide derived from a viral *ets*-1 domain (Antibody A) (lanes 1 and 2), the antibody directed against the common *ets*-1-*ets*-2 domain (Antibody B) (lanes 3 and 4), and the antibody directed against the *ets*-2 domain (lane 5). Competition is shown by preincubation with the *ets*-1 (lane 2) or *ets*-1-*ets*-2 (lane 4) peptides. Immunoprecipitates were fractionated by sodium dodecyl sulfate-polyacryl-amide gel electrophoresis (SDS-PAGE) (12.5% acrylamide) and detected by fluorogra-phy.[34,35] The *arrow* indicates the p135 viral protein. In *lanes 6 through 9* portions of the viral *ets*-1 and human *ets*-2 proteins expressed in *E. coli* were detected by immunoblotting.[36] A portion of the viral *ets*-1 domain (nucleotide 666-1334,[1]) and a cDNA clone of the human *ets*-2 gene (cDNA-14,[1]) have been expressed in *E. coli* and are partially purified.[15] The viral *ets*-1 (lanes 6 and 7) or human *ets*-2 proteins (lanes 8 and 9) were probed by Antibody A (lanes 5 and 6) or Antibody B (lanes 7 and 8). Competition is shown by preincubation of antibodies with their corresponding peptide antigens (lanes 7 and 9). *Arrows* indicate the expressed viral *ets*-1 (lane 6) or human *ets*-2 protein (lane 8). In *lanes 10 through 13* the chicken *ets* protein is immunoprecipitated from [^{35}S]methionine-labeled thymus cells prepared from 2-week-old chickens as described. Competition is shown for Antibody A (lanes 10 and 11) and Antibody B (lanes 12 and 13) by preincubation with the *ets*-1 peptide (lane 11) or *ets*-1-*ets*-2 peptide (lane 13). Relative mobilities of standard proteins are shown on the *sides* of each panel.

Similarly, to characterize the human *ets*-2 protein, we utilized the human tumor-derived cell line, COLO 320 DM. In this study, a 56-KDa protein was specifically immunoprecipitated (FIG. 9, lane 11) from labeled cell lysates using the *ets*-2-specific antibody. In parallel experiments the 56-KDa protein was also able to be

precipitated with common *ets*-1-*ets*-2 domain antibody (FIG. 9, lane 8). However, it should be noted that the *ets*-1 specific antibody does not precipitate this 56-KDa *ets*-2 protein (FIG. 9, lane 6). We can thus conclude that each of these cell lines contain distinct *ets*-1 and *ets*-2 proteins, and that both these proteins can be recognized by antibodies prepared against the common *ets*-1-*ets*-2 domain. Moreover, antisera to a
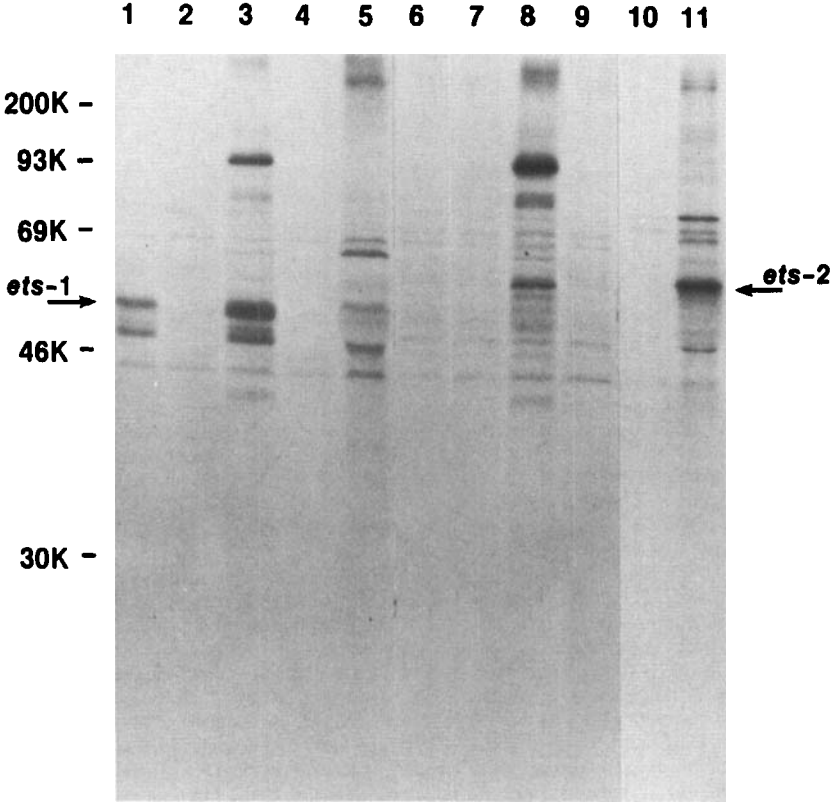
FIGURE 9. Immunoprecipitation of human *ets*-1 and *ets*-2 proteins. [^{35}S]methionine-labeled lysates from the human Burkitt lymphoma cell line, Daudi (*lanes 1 through 5*) or the human COLO 320 DM cells (*lanes 6 through 11*) were immunoprecipitated by Antibody A (lanes 1, 2, 6 and 7), Antibody B (lanes 3, 4, 8 and 9), Antibody C (lanes 5 and 11) or Antibody C pre-immune serum (lane 10). For competition, Antibody A and Antibody B were incubated with the *ets*-1 or *ets*-1-*ets*-2 peptides (lanes 2, 4, 7 and 9). The samples were adjusted to 2×10^7 CPM (TCA insoluble [^{35}S]methionine with RIPA buffer, immunoprecipitated and analyzed by SDS-PAGE and fluorography. The *arrows* indicate the human *ets*-1 (lanes 1 and 3) or the human *ets*-2 (lanes 8 and 11) proteins.

domain unique to *ets*-2 recognized only *ets*-2 protein in human cell extracts and not the *ets*-1 protein, while antisera to an *ets*-1 unique domain recognized only the *ets*-1 protein in human cell extracts.

Given the above observations, we felt it important to establish that the proteins

immunoprecipitated by the different *ets*-2-specific antibodies were similar; this analysis was performed first by one-dimensional peptide mapping using the V-8 protease technique, which showed these maps to be essentially identical (data not shown). To demonstrate that this protein does contain an *ets*-2-related sequence, we employed a two-dimensional peptide-map analysis of the purified ^{35}S-labeled *ets*-2 protein from COLO 320 cells and compared it to the *ets*-2 domain product expressed in an *E. coli* vector. In these studies we found 10 polypeptides shared in common with the human p56 protein and the bacterially expressed *ets*-2 protein.[15] This observation provided direct evidence that the human p56 protein we analyzed shared peptides in common with the authentic *ets*-2-encoded protein expressed in bacteria.

As an approach towards identifying specific functions of the two distinct human *ets* proteins, we fractionated the *ets*-1 and *ets*-2 expressing cells, P3HR-1 and COLO 320 DM, respectively, into subcellular components. Fractionation of [^{35}S]-methionine-labeled COLO 320 DM cells followed by immunoprecipitation, indicated that the human *ets*-2 p56 protein (large arrow, Fig. 10C, lanes 1 and 7) located exclusively in the nucleus. Other proteins immunoprecipitated (small arrows, Fig. 10C, lanes 1 and 7) have been characterized by two-dimensional peptide mapping analysis and shown to share homology with the *ets*-2 p56 protein. It should be pointed out that under the conditions employed for fractionation of COLO 320 DM cells, the *ets*-2 protein was more tightly associated with the nuclear fraction than the "nuclear-associated" c-*myc* gene product.[23]

Similar experiments on the *ets*-1 protein were performed using [^{35}S]-methionine-labeled fractionated human P3HR-1 lymphoid cells. These studies clearly demonstrated that the 51-KDa *ets*-1 protein, unlike the *ets*-2 protein, is located exclusively in the cytoplasm (FIG. 10B, lane 3). To compare this human cellular localization of the *ets* protein to that of the avian cells, we labeled chicken thymus cell and fractionated them as described (FIG. 10, legend). Immunoprecipitation of the avian cell lysates shows that a 51-KDa protein, present in total cell lysates (FIG. 10A, lane 2), was equally distributed between the nuclear and cytoplasmic subfractions. This study indicated that unlike the human protein where the separated products have an *ets*-1 domain that enabled it to localize to the cytoplasm, and an *ets*-2 domain that enabled it to localize to the nucleus, the avian *ets* protein containing both the *ets*-1 and *ets*-2 domains is distributed between the cytoplasm and nucleus.

The human 51-KDa and the 56-KDa proteins described have been demonstrated in our laboratory to be the products of the human *ets*-1 and *ets*-2-encoded domains, respectively. Localization of these proteins, indicate different intracellular compartments for each of these human *ets*-encoded gene products. These segregated subcellular locations suggest further that each of these gene products performs a different function.

An examination of the putative amino acid sequences of the *ets*-2 gene reveals a protein domain containing a region of basic amino acid residues (gly-lys-arg-lys-asn-lys-pro-lys),[10] which is essentially similar to the amino acid residues of the nuclear transport signal of the large tumor antigen of simian virus 40 (pro-lys-lys-lys-arg-lys-val), as well as to that of the T-antigen of polyoma virus (val-ser-arg-lys-arg-pro-arg-pro). It should be pointed out that for nuclear localization signals thus far reported, there is no unique sequence of amino acids specifying nuclear placement; rather there appears to be a sequence of residues encoding a cluster of positively charged amino acids sandwiched between a proline residue and other nonpolar amino acids.[24] This signal region in the *ets*-2 domain may, in fact, prove to be functionally significant for different organisms. It is evident that in higher organisms like mammals, where the *ets*-1 and *ets*-2 domains are separate, each domain remains free to partition to its unique intracellular location. In the chicken, however, where the *ets*-1
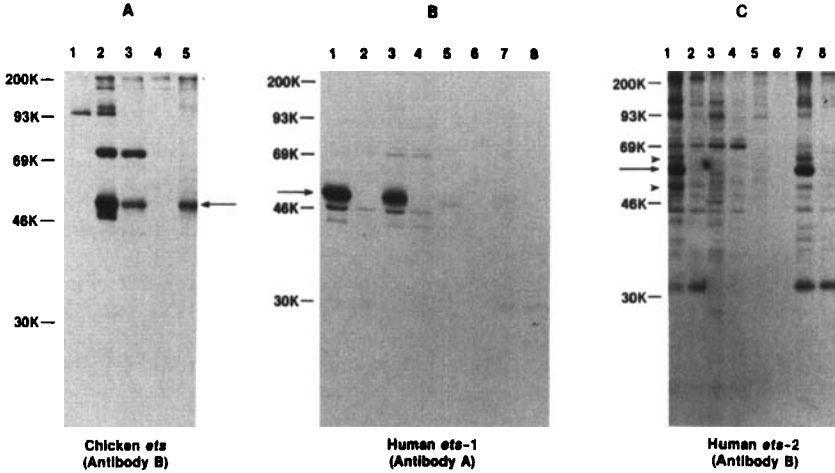
FIGURE 10. Subcellular localization of the chicken *ets*, human *ets*-1 and human *ets*-2 proteins. (A) Thymus cells prepared from 2-week-old chickens were labeled with [^{35}S]methionine (200 μCi/ml, 1 h) and after washes in cold phosphate buffered saline, the cells were gently homogenized in isotonic low salt buffer (20 mM HEPES, 0.25 M sucrose, 5 mM KCl, 5 mM MgCl$_2$, 0.5% NP-40, 0.1% sodium deoxycholate, 1.0% aprotinin, 0.4 mM PMSF, 0.01 mM TLCK, 0.1 mM TPCK) and nuclei were pelleted by centrifugation at 1000 × g for 5 min at 4°C. The crude-cytoplasm was further fractionated by ultracentrifugation (100,000 × g) and the clarified cytoplasm and the membrane pellet were obtained. The nuclear pellet was washed once with the isotonic low salt buffer, after centrifugation at 1000 × g the nuclear wash fraction and the crude nuclei were obtained. The lysis of cells and nuclear isolation were monitored by phase-contrast microscopy. After ultracentrifugation, each fraction was adjusted to RIPA buffer conditions, and extracts (equivalent to 5 × 10^7 cells) were immunoprecipitated with Antibody B. Immunoprecipitation of the total cell extract (Panel A, lanes 1 and 2), cytoplasmic compartment (lane 3), membrane compartment (lane 4) and nuclear compartment (lane 5) are shown. Completion of Antibody B with the *ets*-1-*ets*-2 peptide is in lane 1, and the *arrow* indicates the chicken *ets* protein. (B) Similarly, the P3HR-1 cells were fractionated. The total extract (lanes 1 and 2), the cytoplasmic compartment (lanes 3 and 4), the membrane compartment (lanes 5 and 6) and nuclear component (lanes 7 and 8), each representing 5 × 10^6 cells, were immunoprecipitated with Antibody A. The competition with the *ets*-1 peptide is shown in lanes 2, 4, 6 and 8 and the *arrow* indicates the *ets*-1 protein. (C) The human COLO 320 DM cells were fractionated, as above. The total extract (lanes 1 and 2), cytoplasmic compartment (lanes 3 and 4), membrane compartment (lanes 5 and 6) and nuclear compartment (lanes 7 and 8), each presenting 5 × 10^6 cells, were immunoprecipitated with Antibody B. Competition with the *ets*-1-*ets*-2 peptide is shown in lanes 2, 4, 6 and 8 and the *arrow* indicates the *ets*-2 protein.

and *ets*-2 domains are in the same protein, the localization of the *ets* gene product may depend on other factors. Unlike the variant locations of human *ets* proteins, we find that the chicken *ets* gene product is equally distributed between the cytoplasmic and nuclear subcellular fractions; this observation would seem to be consistent with the known genetic organization of both *ets* domains in the avian genome.[9,13] It is possible, too, that the avian *ets* protein, in response to some physiological event, shuttles between the cytoplasm and nucleus, whereupon each domain is able to exert its distinctive activity.

Both *ets*-1 and *ets*-2 domains may be of potential interest in oncology. The transposition of these genes in nonrandom translocations found in specific leukemias,

as well as in their associated altered expression, suggests that both *ets*-1 and *ets*-2 genes may be involved in human hematopoietic neoplasias.[25] Differential expression of *ets* proteins could provide useful molecular markers for the characterization of hematopoietic tumors.

Having established that the human genome contains two distinct loci with homology to the viral *ets* gene, Hu-*ets*-1 and Hu-*ets*-2, made it possible to precisely map their locations onto their respective human chromosomes. Employing both somatic cell hybrid studies and *in-situ* chromosomal techniques, we have localized the human proto-*ets*-1 and proto-*ets*-2 domains to the human chromosomes, 11q23 and 21q22, respectively.[26]

A number of leukemias of myelomonocytic lineage show chromsome abnormalities in the very region where the human *ets* loci reside. In fact, the 11q23 region where the Hu-*ets*-1 locates is known to be involved in a number of translocations associated with acute myeloid leukemias. We have, therefore, focused primarily on the t(4;11)(q21;q23) translocation, an aberration that is associated with a rare infant leukemia of the undifferentiated type (AUL) thought to be congenital, and could perhaps involve the Hu-*ets*-1 locus. Similarly, we investigated the t(8;21)(q22;q22) translocation, associated with a subtype of acute myelogenous leukemia (AML-M2) involving the 21q22 chromosomal region, where Hu-*ets*-2 is located (FIG. 11).
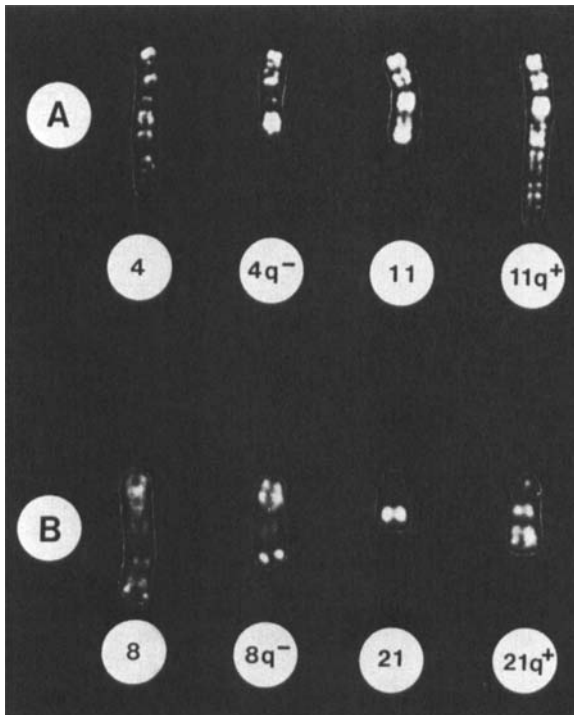

FIGURE 11. Partial karyotype analysis showing the normal and recombinant chromosomes resulting from: (A) the reciprocal translocation t(4;11)(q21;q23), and (B) the reciprocal translocation t(8;21)(q22;q22).

From Southern blot studies using genomic DNA samples derived from cell hybrids containing the recombinant chromosomes derived from these translocations, we have found that the Hu-*ets*-1 gene does in fact translocate from chromosome 11 to 4, while the Hu-*ets*-2 gene was found to transpose from chromosome 21 to 8.[27] Using *in-situ* hybridization, other workers have shown, with gene probes for γ-interferon and v-*ets* by hybridizing them to metaphase chromosomes obtained from three patients with AML having the chromosomal translocation t(9;11)(p22;q23), that the proto-*ets*-1 gene was translocated from chromosome 11 to the short arm of chromosome 9 adjacent to the interferon genes.[28] Such studies reinforce the supposition that juxtapositioning of the proto-*ets* genes next to other cellular genes may lead to their dysregulation. Further, the translocations we have observed appear to affect the transcription patterns of both Hu-*ets* genes; these were confirmed by Northern blot studies on RNA derived from leukemic and control samples, as well as from continuous cell lines derived from patients. In such comparative studies, albeit on limited samples, we have found that in t(4;11) leukemic cells significantly lower levels of Hu-*ets*-1 mRNA were present. Similarly, studies on t(8;21) AML samples revealed that not all the species of Hu-*ets*-2 mRNA known to be present in normal lymphocytes and other human cells were present. Particularly absent, or markedly reduced, was the larger 4.7-kb species of human *ets*-2 mRNA, suggesting that this alteration of expression of human *ets* mRNA in leukemia patient samples may arise as a consequence of the specific position effects sustained (FIG. 12).

The significance of these studies and their specificity was further reinforced by utilizing our Hu-*ets*-1 probe to show that the *ets*-1 gene was not transposed in the (11;22)(q12;q23) translocation of Ewing sarcoma, in contrast to the (4;11) translocation noted previously. However, in neither case was there any evidence that the human *ets*-1 or *ets*-2 genes were rearranged, although the precise breakpoints and their location relative to these proto-oncogenes remains to be determined. Such proto-*ets* gene relocations may well play a critical role in altering the regulation of these unique genes for certain specific leukemias; any altered products arising from such translocations may serve as useful diagnostic or prognostic markers in these specific pathologies.[29]

The 21q22 region or, specifically, the 21q22.3 region, where Hu-*ets*-2 has been physically and genetically mapped, seems to be relevant not only to cancer but to other human cytogenetic pathologies as well. This region is the precise region that when trisomic appears to confer the Down's syndrome (DS). DS afflicts 1 out of 600 newborns, and such individuals display a number of dismorphic features and developmental abnormalities, including mental retardation.[30] The causes of trisomy of the 21q22.3 region are mainly due to the trisomy of the entire chromosome 21. Very rarely, but significantly, a microduplication of the q22.3 region or inheritance of this region attached to an autosome (partial trisomy) may be the cause of the DS phenotype. Such rare individuals, therefore, may prove to be very informative for defining the minimal genetic region required for the appearance of DS. Since we know that the Hu-*ets*-2 gene locates in this region, studies with this probe may become quite valuable for the initiation of molecular cloning studies necessary to identify and isolate the entire region responsible for DS. Furthermore, preliminary data shows three copies of the *ets*-2 gene in some of these rare DS patients using an *ets*-2-specific probe cloned in our laboratory.[31] Significantly, DS patients show a higher incidence of leukemia, as well as precocious neurological signs of Alzheimer's disease.[30] Having a specific antibody for the human *ets*-2 proteins available will permit us to evaluate and precisely quantitate the expression of this gene in both Down's syndrome and Alzheimer's disease.

Finally, restriction fragment-length polymorphisms (RFLPs) for the *ets*-2 gene have been used as genetic markers to study whether or not the genetic constitution
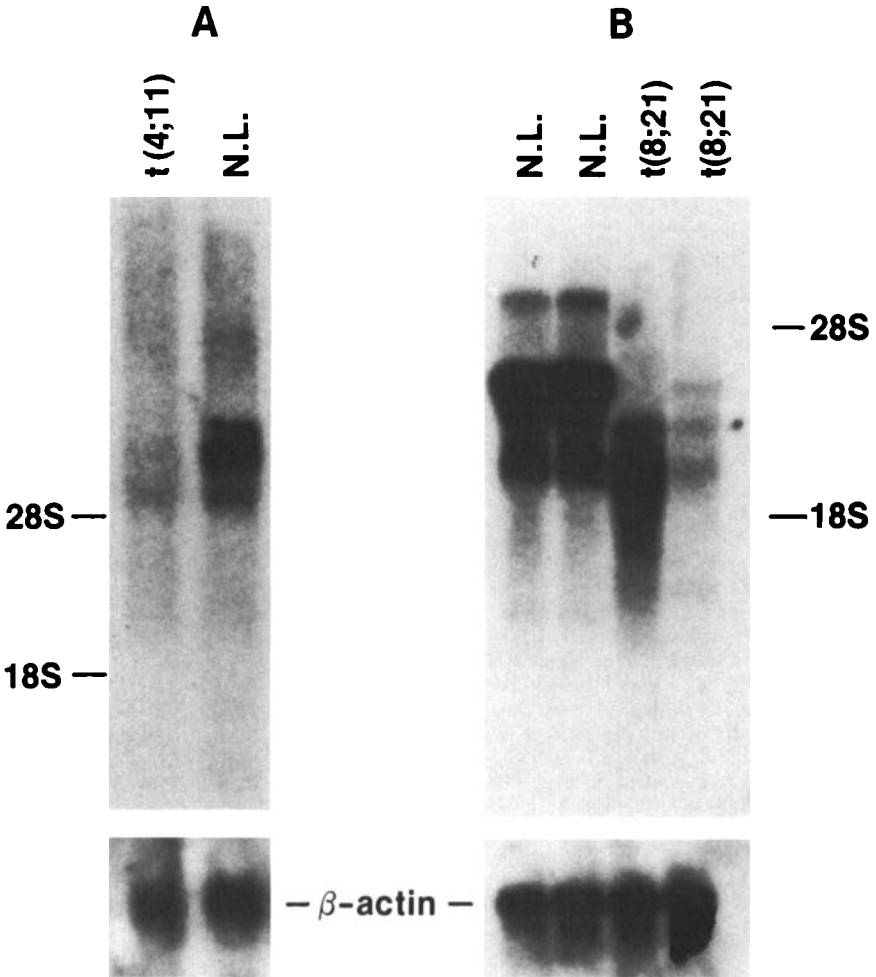
FIGURE 12. Northern blot analysis of total RNA extracted from: (A) blast cells derived from a t(4;11) AUL patient and normal lymphocytes (N.L.), and (B) blast cells derived from two t(8;21) AML-M2 patients and normal lymphocytes. In all cases, total RNA was extracted by the guanidinium/cesium chloride method and was resolved on 1.2% formaldehyde-agarose gels. Hybridization was performed under stringent conditions (2b) with: (A) *ets*-1 (RD6K) and (B) *ets*-2 (H33) probes. The same blots (*bottom*) were rehybridized using an α-actin cDNA probe in order to compare relative amounts of RNA in these samples.

(haplotype) of the chromosome 21 that undergoes normal, meiotic disjunction is different from the haplotype of chromosome 21 that is not undergoing disjunction and therefore predisposing to the trisomy associated with Down's syndrome. The results of our extensive survey with the Hu-*ets*-2 probe, performed on families with a Down's syndrome child, shows that all examples of chromosome 21 are genetically similar, thus indicating that haplotype analysis of chromosome 21 is not applicable to the

prevention of this syndrome.[32] This finding is important since on the basis of a previous study the clinical relevance of similar chromosomal haplotype tests was suggested.[33] According to our experience, such tests should be viewed with extreme caution and reservation.

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