Alternative Splicing within the Chicken *c-ets-1* Locus: Implications for Transduction within the E26 Retrovirus of the *c-ets* Proto-Oncogene

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Two overlapping *c-ets-1* cDNA clones were isolated which contained the α and β genomic sequences homologous to the 5' end of *v-ets* not detected in the previously described *c-ets* RNA species or proteins. Nucleotide sequencing demonstrated that these cDNAs corresponded to the splicing of α and β to a common set of 3' exons (*a* through *F*) already found in the *p54^{c-ets-1}* mRNA. They contained an open reading frame of 1,455 nucleotides which could encode a polypeptide of 485 amino acids with a predicted molecular mass of 53 kilodaltons. However, when expressed in COS-1 cells, the cDNAs directed the synthesis of a protein with an apparent molecular mass in sodium dodecyl sulfate-polyacrylamide gel electrophoresis of 68 kilodaltons, *p68^{c-ets-1}*, comigrating with a protein expressed at low levels in normal chicken spleen cells. These two proteins were shown to be identical by partial digestion with protease V8. Northern (RNA) blot hybridization analysis with the *p68^{c-ets-1}*-specific sequence and RNase protection experiments showed that the corresponding mRNA was expressed in normal chicken spleen and not in normal chicken thymus or in various T lymphoid cell lines. Thus, two closely related proteins, having distinct amino-terminal parts, are generated within the same locus by alternative addition of different 5' exons, α and β or *I⁵⁴*, respectively, onto a common set of 3' exons (*a* to *F*). Finally, we demonstrate that an aberrant splicing event between a cryptic splice donor site in *c-myb* exon E6 and the normal splice acceptor site of *c-ets-1* exon α is involved in the genesis of the E26 *myb-ets* sequence.

The avian acute leukemia virus E26 induces a mixed erythroid-myeloid leukemia and transforms cells of both erythroid and myeloid lineages in vivo and in vitro (2, 22, 35, 41). The 5.7-kilobase (kb) E26 genomic RNA contains two distinct cell-derived oncogenes, *v-myb* and *v-ets*, expressed, together with a partial viral *gag* gene, as a 135-kilodalton (kDa) nuclear fusion protein, P135^{*gag-myb-ets*} (6, 23, 29, 40).

We previously reported that the chicken cellular sequence from which *v-ets* presumably arose, *c-ets*, is a contiguous locus split into two domains (6.0 and 18.0 kilobase pair [kbp], respectively) separated by ca 40.0 kbp of *v-ets*-unrelated DNA (15). These studies were further consolidated by the characterization and nucleotide sequencing of a *c-ets* cDNA clone able to encode the major chicken *ets* protein *p54^{c-ets}* (8, 19) by transient expression in COS-1 cells (M. Duterque-Coquillaud, D. Leprince, A. Flourens, C. Henry, J. Ghysdael, B. Debuire, and D. Stehelin, *Oncogene Res.*, in press). All these data can be summarized as follows.

(i) The 3' domain (18.0 kbp) contains six regions of homology to *v-ets*, named *a* to *f*. Regions *a* to *e* were homologous between the viral and cellular sequences, except for five point mutations, two silent and three chemically conservative (Duterque-Coquillaud et al., in press). By contrast, the last region, *f*, is a truncated and altered form of a *c-ets* cellular exon named *F*. The *v-ets*- and *c-ets*-encoded proteins differ in their carboxy-terminal parts, since the last 13 codons in the cellular exon are replaced by 16 codons of unknown origin in the viral sequence (15; Duterque-Coquillaud et al., in press).

(ii) The 40.0-kbp domain unrelated to *v-ets* contains the

first coding exon of the *p54* cDNA clone. This exon (named *I*) which, for clarity, is referred to as *I⁵⁴*, should be fused to exons *a* to *F* to yield the *p54^{c-ets}*-encoding sequence (Duterque-Coquillaud et al., in press).

(iii) The 5' domain accounts for 223 nucleotides at the 5' part of *v-ets* and is composed of two sets of sequences, named α and β , presenting the structural properties of typical eucaryotic exons (15). This domain is not transcribed into the major 7.5-kb *c-ets* RNA species identified in lymphoid cells such as MSB1, translated into *p54^{c-ets}* (15, 16; Duterque-Coquillaud et al., in press), or translated into the more distantly related *p58* to *p63* proteins found in macrophages (18) and originating from another locus (7). However, to yield the viral *v-ets* sequence, α and β should be fused by splicing to exon *a* (15). Similarly, the *I⁵⁴* exon should be fused to exon *a* to yield the *p54^{c-ets}*-encoding sequence (Duterque-Coquillaud et al., in press).

(iv) The human homologs of the *ets* oncogene consist of at least two distinct domains located on different chromosomes: ETS-1 on chromosome 11 (13, 60, 61) and ETS-2 on chromosome 21 (60, 61). Recently a new member of the *ets* oncogene family, *erg*, has been identified (42). The chicken genome also harbors two loci related to *v-ets*: a DNA locus highly related to *v-ets* and encoding *p54^{c-ets-1}*, known as chicken *c-ets-1* (15, 19; Duterque-Coquillaud et al., in press), and a recently characterized locus only distantly related to *v-ets*, encoding the *p58* to *p64* proteins expressed in macrophages, named chicken *c-ets-2* (7, 18).

In this report, we demonstrate that alternative splicing events are operative in the chicken *c-ets-1* locus to generate either the mRNA species coding for *p54^{c-ets-1}* or a previously undetected mRNA species containing α and β . We cloned

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two distinct overlapping cDNA clones differing from *v-ets* only in their 3' end. These cDNAs correspond to the expected splicing of α and β sequences to the common set of 3' exons (*a* to *F*). These cDNAs, when expressed in COS-1 cells, directed by synthesis of a novel p68^{c-ets-1} protein closely related to p54^{c-ets-1}, but having a distinct amino-terminal part and detected at low levels in normal chicken spleen cells.

Finally, we demonstrate that an aberrant splicing between a cryptic splice donor site located in *c-myb* exon E6 and the normal splice acceptor site of *c-ets-1* exon α is involved in the genesis of the E26 *myb-ets* sequence.

MATERIALS AND METHODS

cDNA cloning and isolation of *c-ets-1* cDNA clones. An SPF chicken (two animals; SPAFAS) spleen cell cDNA library in lambda gt10 prepared by modification of the Hoffman-Gubler procedure was kindly provided by K. Strebhardt and J. Mullins. An SPF 11-day-old chicken embryo cDNA library in lambda gt11 was a kind gift of J. Sap and B. Vennstrom (47). Both libraries were screened by hybridization to *v-ets*-specific probes. Hybridization was carried out at 41°C in 50% (wt/vol) formamide–3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0])–5× Denhardt solution–100 µg of denatured salmon sperm DNA per ml–0.1% sodium dodecyl sulfate (SDS), and filters were washed at 50°C in 0.1× SSC–0.1% SDS. Approximately 400,000 recombinant plaques were screened from each library as previously described (Duterque-Coquillaud et al., in press).

Nucleotide sequence analysis. The DNA sequence was determined by the dideoxy chain termination method (46) after subcloning of specific restriction fragments into the polylinker restriction sites of M13 bacteriophage derivatives mp10, mp11, mp18, and mp19 (33).

COS cell transfection. The *EcoRI* sites of the *c-ets-1* cDNA clones were converted to blunt ends by using the Klenow fragment of DNA polymerase I and subcloned into the *SmaI*-digested pSVL COS expression vector (Pharmacia) in the correct orientation with respect to the simian virus 40 late promoter of transcription.

Plasmid DNA (10 µg) was transfected into 10⁶ COS-1 cells (20) in a 100-mm dish by the DEAE-dextran method (52) with the addition of a chloroquine treatment (30) as previously described (Duterque-Coquillaud et al., in press).

Radiolabeling of cells, immunoprecipitation, and peptide-mapping analyses. Cells were labeled with L-[³⁵S]methionine in methionine-free Eagle medium supplemented with 5% dialyzed calf serum and lysed by boiling for 5 min in a 1% solution of SDS. After dilution with 10 volumes of 150 mM NaCl–1 mM EDTA–10 mM Tris hydrochloride (pH 7.4)–1% Triton X-100–0.5% sodium deoxycholate–1% Trasylol, the lysates were centrifuged at 100,000 × *g* for 60 min and immunoprecipitations were carried out as described previously with anti-*ets* sera (16, 19). Before immunoprecipitation, the spleen cell lysates were incubated with 50 µl of 50% protein A-Sepharose beads (suspended in radioimmunoprecipitation buffer) for 30 min at 4°C with gentle shaking to eliminate the heavy chain of avian immunoglobulin (ca. 68 kDa) present in B lymphocytes of chicken spleens.

Partial digestion by *Staphylococcus aureus* V8 protease in gel slices containing [³⁵S]methionine-labeled proteins was done essentially as described by Cleveland et al. (9).

RNA blot analysis. Total cellular RNAs were prepared by the guanidine thiocyanate procedure, fractionated by electrophoresis on a 1% agarose–formaldehyde gel, and trans-

ferred to Hybond membranes (Amersham Corp.). Transfer, hybridization, and washing were done as recommended by the manufacturer.

Nuclease protection experiment. The *EcoRI*-*Bgl*II 566-bp fragment from the p68-2 cDNA clone was subcloned in pGEM4. After linearization by *EcoRI*, an RNA probe complementary to the mRNA (cRNA) was synthesized by using T7 phage polymerase (Promega Biotec). Total RNA (20 µg) or 5 µg of *Escherichia coli* tRNA was mixed with 5 × 10⁵ cpm of [³²P]cRNA (specific activity, 10⁹ dpm/µg) in 30 µl of 80% formamide–400 mM NaCl, 1 mM EDTA–40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.5). The mixture was heated at 85°C for 5 min and incubated for 4 h at 37°C and then for 10 h at 30°C (27). After hybridization, 300 µl of RNase digestion buffer (5 mM EDTA, 300 mM NaCl, 10 mM Tris hydrochloride [pH 7.5]) containing 30 µg of RNase A per ml and 2 µg of RNase T1 per ml was added, and the mixture was incubated for 30 min at 30°C. The nucleases were removed by digestion with proteinase K, extracted with phenol-chloroform, and ethanol precipitated with 5 µg of tRNA as carrier. Protected fragments were analyzed on 6% sequencing gels. The exact sizes of the protected fragments were determined by comparison with adjacent sequencing reactions.

RESULTS

Isolation and sequencing of cDNA clones containing α and β *v-ets* sequences. We previously reported the cloning of p54^{c-ets-1} cDNAs from chicken embryo or spleen cell cDNA libraries (Duterque-Coquillaud et al., in press). These libraries were screened at high density with a probe corresponding to α and β *v-ets* sequences (*Sau*3a-*Hpa*I 250-bp fragment) and containing 25 nucleotides from exon *a* also present in the p54^{c-ets-1} cDNA clone. We focused our attention on two clones (known as lambda *ets* 68-1 and lambda *ets* 68-2) that were negative with an *EcoRI*-*Cl*aI 150-bp probe specific for p54^{c-ets-1} cDNA clones (Fig. 1) (Duterque-Coquillaud et al., in press). Lambda *ets* 68-1 (obtained from the chicken embryo cell cDNA library) contained two *EcoRI* fragments: a ca. 1.5-kbp fragment hybridizing with *v-ets* probes, and a 0.8-kbp fragment negative with these probes and presumably representing 3' untranslated sequences (see below). Lambda *ets* 68-2 (obtained from the spleen cell cDNA library) contained a single ca. 1.4-kb *EcoRI* fragment hybridizing with *v-ets* probes (Fig. 1). These data are in agreement with our previous reports that a true *EcoRI* site is present in the *c-ets-1* cellular exon *F* and that some *EcoRI* sites escaped methylase treatment during the construction of the library (15; Duterque-Coquillaud et al., in press). The DNA of these two clones was sequenced by using the strategy outlined in Fig. 1, and the results are illustrated in Fig. 2.

Identification of a novel p68^{c-ets-1} protein. The composite *c-ets-1* mRNA sequence of the *ets*-68 cDNA clones was determined by the dideoxy chain-terminating method of Sanger et al. (46). The longest open reading frame starting with a methionine codon at position 70 in the nucleotide sequence is expected to encode a 485-amino-acid polypeptide with a predicted molecular mass of 53 kDa (Fig. 2). We transiently expressed these cDNA clones in COS-1 cells as previously described (Duterque-Coquillaud et al., in press). The *v-ets*-related *EcoRI* fragment of each cDNA clone was converted to blunt ends and cloned into the *SmaI* site of the simian virus 40-derived expression vector pSVL. After transfection into COS-1 cells, the resulting polypeptides were characterized by immunoprecipitation with anti-*ets*

FIG. 1. Structure of the chicken $p68^{c-ets-1}$ cDNA clones compared with the chicken genomic *c-ets-1* locus. (A) A schematic organization of the chicken *c-ets-1* locus (■, cellular exons transduced by the virus; □, nontransduced exons) is shown at the top. Nomenclature is as in reference 15 and Duterque-Coquillaud et al., in press. Only some relevant restriction sites, *Cla*I, *Eco*RI (E), *Hind*III (HdIII), and *Hpa*I are shown. The restriction map of the two *c-ets* cDNA clones is represented below: ▨, 5' and 3' untranslated regions, the open portion, the *c-ets-1* coding sequence, *E, *Eco*RI sites generated by the cloning. (B) Detailed restriction map of the λ p68-2 cDNA clone showing cleavages for *Bgl*II (Bg), *Eco*RI (E), *Hind*III (Hd), *Pst*I (P), *Pvu*II (PII), and *Sau*3A (S). The arrows beneath this restriction map denote the sequencing strategy used. The *Eco*RI-*Hpa*I and *Hind*III-*Eco*RI fragments of λ p68-1 have also been sequenced.

sera and electrophoresis in an SDS-polyacrylamide gel (19). A predominant polypeptide of approximately 68 kDa, as well as bands of lower molecular mass (in particular a ca. 60-kDa polypeptide), not detectable in mock-transfected COS-1 cells (data not shown; Duterque-Coquillaud et al., in press), was specifically immunoprecipitated by the previously described anti-*etsA* serum (Fig. 3A, lanes 1 and 4), as well as by the anti-*etsB* serum (Fig. 3A, lanes 3 and 6) directed against the α - β -encoded domain of P135^{gag-myb-ets} (16). This immunoprecipitation is specific, since it is abolished by anti-*ets* sera blocked with an excess of the bacterial immunogen (Fig. 3A, lanes 2 and 5).

The apparent discrepancy between the predicted molecular mass (53 kDa) and the apparent molecular mass observed in the SDS-polyacrylamide gel (68 kDa) could reflect some intrinsic properties of *ets* proteins such as their high content of proline residues. Such a discrepancy was previously observed, although to a lesser extent (48 instead of 54 kDa), with the cDNA encoding $p54^{c-ets-1}$ (Duterque-Coquillaud et al., in press). We therefore compared the cDNA-encoded proteins in COS-1 cells with the cellular *ets* proteins encoded in normal chicken cells. Although only the $p54^{c-ets-1}$ protein can be observed in thymus (data not shown) or in MSB-1 T lymphoid cells (Fig. 3A, lane 10), a novel $p68^{c-ets-1}$ protein similar in an SDS-polyacrylamide gel electrophoresis one-dimensional gel to the one observed in transfected COS-1

cells can be detected at low levels in normal spleen cells (Fig. 3A, lanes 7 to 9). To further substantiate the relationship between these two proteins, we compared the one-dimensional cleavage patterns obtained from these polypeptides after partial digestion with *S. aureus* V8 protease, a procedure particularly suited to demonstrating the extensive sequence homology between polypeptides (9). The results (Fig. 3B) demonstrate that the COS-1 cDNA-encoded and the splenic cellular proteins $p68^{c-ets-1}$ are identical. In addition, the spleen, the organ from which one of the cDNA libraries was constructed, expressed considerably higher levels of $p54^{c-ets-1}$ (Fig. 3A, lanes 7 and 8) as previously described by Chen (8). We thus conclude that the cDNAs we sequenced encompass an intact coding element for *c-ets-1*. This was further confirmed by nucleotide sequencing, demonstrating that the three reading frames upstream of the ATG codon 70 are closed by termination codons (Fig. 2). However, from our data it is impossible to definitively authenticate the initiation codon for $p68^{c-ets-1}$. There are actually five in-frame methionine codons close to each other at the 5' end of the cDNA clone (Fig. 2). Although none of the flanking sequences of these ATG codons shows a perfect match with the Kozak consensus sequence [CC(A/G)CATGG] (26), two of them are presumably not used (positions 85 and 175 in Fig. 2), since their context appears very unfavorable, with a T instead of a purine at position -3 of the Kozak consensus sequence, a feature believed to be important for initiation of translation (26). Thus, we do not know which of the three remaining ATG codons (positions 70, 73, and 139 in Fig. 2) in a more favorable context, if any, is the true initiation codon. The multiple products encoded by the $p68^{c-ets-1}$ cDNA could result from alternative initiations at other internal in-frame ATG codons (53).

Relation of the $p54^{c-ets-1}$ cellular protein. We aligned the nucleotide sequences of the cDNAs coding for $p68^{c-ets-1}$ and $p54^{c-ets-1}$ with that of the previously described genomic exons α , β , and a (15; Duterque-Coquillaud et al., in press). These data (summarized in Fig. 4) were used to bolster our previous suggestions regarding both the nature and origin of the α and β sequences and alternative splicing events within the *c-ets-1* locus (Duterque-Coquillaud et al., in press). In addition to some point mutations, the sequence encoding $p54^{c-ets-1}$ and the sequence reported here diverge upstream of nucleotide 283 (Fig. 2 and 4). This nucleotide corresponds exactly to the leftward boundary of exon a . Thus, this cellular exon is a common acceptor exon, which can be fused by splicing either to the first $p54^{c-ets-1}$ -encoding exon, I^{δ} , or to the α and β $p68^{c-ets-1}$ -encoding exons as shown by the nucleotide sequence (Fig. 4). Taken together, these data clearly demonstrate that an alternative splicing mechanism within the *c-ets-1* locus generates two highly related proteins having distinct amino-terminal parts: $p54^{c-ets-1}$, expressed at high levels in chicken thymus, and $p68^{c-ets-1}$, detected so far only in chicken spleens.

Expression and tissue specificity of the $p68^{c-ets-1}$ mRNA. We next determined the size of the mRNA species encoding $p68^{c-ets-1}$. We first prepared a probe specific for $p68^{c-ets-1}$ and devoid of common sequences. The *Eco*RI-*Pst*I 190-bp fragment of λ p68-2 (Fig. 1B and 4) was subcloned into pGEM4. Using this fragment as a probe, we analyzed total RNA prepared from spleens obtained from 14-day-old embryos or 2-day-old chickens, from normal chicken thymus, from MSB-1 (a T lymphoid cell line transformed by Marek disease virus), and from different clones of lymphoid cells transformed by reticuloendotheliosis virus and blocked at various immature stages of differentiation (3). High levels of

FIG. 2. Nucleotide sequence of 1.5 kbp of the *c-ets-1* cDNA clone deduced from that of cDNA clones p68-1 and p68-2. The translation of this sequence in the long open reading frame, which extends from the first in-frame ATG codon at position 70 to the termination codon TGA at position 1524, is shown. Termination codons upstream of the first ATG are underlined. Only the nucleotides and amino acids in *v-ets* that differ from *c-ets-1* are indicated and boxed.

was in agreement with our previous results (15, 16) and was significant since the same blot hybridized with a common *ets* probe encompassing exons *a* to *F* (1.2-kbp *HpaI-EcoRI* fragment) revealed the major 7.5-kb *c-ets-1* RNA species (Fig. 5B, lanes 1 to 7). The 2.2- and 1.5-kb RNA species,

FIG. 3. $p68^{c-ets-1}$ transiently expressed in COS-1 cells transfected by $p68^{c-ets-1}$ cDNA is similar to a cellular protein expressed in spleen cells. (A) COS-1 cells were transfected with 10 μ g of plasmid DNA, labeled 72 h later with [35 S]methionine, and analyzed by immunoprecipitation with anti-*etsA* serum (lanes 1 and 4), anti-*etsA* adsorbed with an excess of bp17^{MS2-etsA} (lanes 2 and 5), and anti-*etsB* serum directed against the α - β -encoded domain of P135^{gag-myb-ets} (lanes 3 and 6) (16). Cell suspensions obtained from the spleen of a 2-day-old chicken (lanes 7 through 9) were treated in the same way after preincubation with protein A-Sepharose beads (see Materials and Methods), as well as MSB-1 T lymphoid cells. The immunoprecipitation of $p68^{c-ets-1}$ by anti-*etsB* sera, which is difficult to see in the figure (lane 9), was clearly visible on the original autoradiogram. Prestained protein molecular weight standards (Bethesda Research Laboratories, Inc.) were run on the SDS-polyacrylamide gel and used to determine the sizes of the immunoprecipitated proteins (not shown). (B) One-dimensional V8 protease mapping of COS-1 cDNA-encoded and normal $p68^{c-ets-1}$ proteins. Spleen cells were labeled with 2 mCi of L-[35 S]methionine per ml and treated as described in Materials and Methods. COS-1 cells were transfected with p68-2 cDNA clone and labeled 72 h later with 0.5 mCi of L-[35 S]methionine. Labeled proteins were excised from gels similar to those in panel A and run on a 15% polyacrylamide gel after partial digestion for 30 min with either 20 or 200 ng of *S. aureus* V8 protease. Lanes: Sp, proteins obtained from spleen cells; Cos-1, proteins obtained from transfected COS-1 cells. The positions of prestained protein molecular weight standards are shown on the right (in thousands).

difficult to see on this autoradiogram, were clearly visible on a longer exposure (data not shown).

Interestingly enough, the major $p68^{c-ets-1}$ RNA was also 7.5 kb in size (Fig. 5A, lanes 6 and 7) and could not be resolved from the $p54^{c-ets-1}$ mRNA species even by extensive electrophoresis in low-concentration agarose gels (data not shown). The existence of two distinct proteins encoded by two distinct cDNAs containing alternative 5' exons upstream of the common exon *a* strongly supports the idea that the 7.5-kb RNA observed in spleen cells was in fact a doublet. To demonstrate this, we performed RNase protection experiments (27). We subcloned in pGEM4 an *EcoRI*-*BglII* 566-bp fragment from the p68-2 cDNA clone. The probe (608 nucleotides) complementary to the mRNA (cRNA) was transcribed from the T7 polymerase promoter and encompassed 42 nucleotides from the plasmid promoter and polylinker, 305 nucleotides from the common exons (*a* and part of *b*) and 261 nucleotides corresponding to the $p68^{c-ets-1}$ -specific 5' exons (Fig. 5C, bottom). RNA species specific for $p54^{c-ets-1}$ should protect 305 nucleotides of the cRNA (common fragment) from RNase digestion. Such RNase-resistant hybrids were formed with RNAs extracted from thymus or lymphoid cell lines (Fig. 6C, lanes 3 to 5). In contrast, with RNAs prepared from spleen cells, fully protected fragments were observed in addition to the 305-nucleotide hybrids (Fig. 5C, lane 6).

The apparent ratio (ca. 1:20) in Fig. 5C, lane 6, between the fully protected fragments ($p68^{c-ets-1}$ specific) and the common fragments ($p68^{c-ets-1}$ and $p54^{c-ets-1}$) seemed to be in good correlation with the relative ratio of $p54^{c-ets-1}$ and $p68^{c-ets-1}$ proteins synthesized in spleen cells (Fig. 3A, lane 7).

Thus, a single transcriptional unit directs the synthesis of two similar-sized mRNAs (7.5 kb) with differential expression and encodes two highly related proteins (Fig. 7, top row).

Transduction of *c-ets*. Sequences of *v-ets* and of the $p68^{c-ets-1}$ cDNA are highly homologous except for their carboxy-terminal parts. The cellular protein $p68^{c-ets-1}$, as well as $p54^{c-ets-1}$, diverges from the viral protein in the last 13 codons. Seven point mutations, five of which are conservative, are found between $p68^{c-ets-1}$ and *v-ets* (Fig. 2). These reflect the differences observed between the common set of cellular exons (*a* to *F*) and their viral homologous sequences. Otherwise, $p68^{c-ets-1}$ and the *v-ets* sequence appear colinear, especially in their 5' parts (Fig. 4). We conclude that $p68^{c-ets-1}$, rather than $p54^{c-ets-1}$, should be considered the cellular progenitor of the viral *ets* oncogene. Focusing now on the 5' end of the p68 cDNA, its nucleotide sequence appears colinear with that of *v-ets* until nucleotide 59, which corresponds to the leftward boundary of exon α on one hand and to the *myb-ets* boundary in the E26 genome on the other. This observation deserves two comments. First, if we assume that the ATG codon 70 is not the true initiation codon, 11 nucleotides or more which are noncoding in $p68^{c-ets-1}$, since they are located upstream of the first ATG codon, are converted into coding sequences in the viral protein (Fig. 4). Second, the observation that the *myb-ets* boundary corresponds exactly to the boundary of *c-ets-1* exon α might not be coincidental and suggests a region of splicing. Therefore, we compared the *myb-ets* sequence with *c-ets-1* and *c-myb* genomic sequences and searched for a putative splice donor consensus sequence in this region of *c-myb* (Fig. 6). In fact, the rightward recombination point in E26 *v-myb*, although located in the middle of a cellular *c-myb* coding exon named E6 (24), displays salient homology (6 of 10 nucleotides conserved) with a splice donor site consensus sequence (CAAGGtagagt) (36) (Fig. 6).

In conclusion, the transduction of the *c-ets* proto-oncogene presumably occurred via an abnormal splicing event between a cryptic splice donor site in *c-myb* exon E6 and the normal splice acceptor site of *c-ets-1* exon α as diagrammat-

FIG. 4. Comparison of the two-*c-ets-1* cDNA types with *v-ets* and their corresponding genomic clones. The sequence of the 5' end of the p68^{c-ets} cDNA clones is aligned with that of *v-ets* (39, 40), p54^{c-ets-1} cDNA (Duterque-Coquillaud et al., in press), and the relevant parts of genomic α, β, and a exons (15; Duterque-Coquillaud et al., in press). Asterisks (*) indicate identical sequences shared between the sequences. The putative ATG codons are underlined, whereas the termination codons are boxed. The intron consensus splice junctions (36) are indicated by an arrow with s. a. or s. d. The conserved GT and AG nucleotides are written in lower-case letters and underlined. Numbering in the right-hand column is similar to that used in Fig. 2.

ically outlined at the bottom of Fig. 7. In addition, the *myb-ets* boundary is not located at the second G or the GGC codon as previously suggested (39, 40), but, rather, at the first G of this codon (Fig. 6).

DISCUSSION

To understand how a cellular proto-oncogene, *c-ets-1*, has been converted into an activated viral oncogene, *v-ets*, we have compared the cellular *ets* coding sequences isolated from both genomic DNA (15) and cDNA cloning (Duterque-Coquillaud et al., in press) to the viral *ets* sequence. These studies confirmed and extended the relationship between the major *c-ets-1*-encoded protein, p54^{c-ets-1}, and the *ets*-encoded domain of the viral transforming protein P135^{gag-myb-ets} (19). However, the nature and origin of the α and β sequences remained obscure, since they were homologous to the 5' part of *v-ets* and displayed structural features of eucaryotic exons, but were never detected in *c-ets-1* RNA species or proteins. These sequences deserved special attention, since they represented the leftward recombination point of *c-ets-1* with *c-myb* in E26. In this communication we report the cloning and sequencing of a new *c-ets-1* cDNA

which allowed us to draw several conclusions regarding both the transcription of the cellular *c-ets-1* locus and the transduction of *c-ets*.

First, these results clearly demonstrate that an alternative splicing mechanism is operative in the chicken *c-ets-1* locus. Alternative splicing has been found in a number of DNA viruses and in cellular genes, including several proto-oncogenes (reviewed in reference 28). In the proto-oncogenes, alternative splicing (32, 48, 59) could affect the level of gene expression by selection of different promoters or could generate different proteins (i) by insertion of small amino acid sequences as in the examples of the brain-specific form of pp60^{c-src} (31) and chicken *c-mil* (14) or (ii) by addition of different 5' exons onto a common set of 3' exons as in the examples of the murine (1) or human (49) *c-abl* genes and the chicken *c-myb* gene (17, 45). The alternative splicing in chicken *c-ets-1* that we report here is an example of the latter case and yields two highly related proteins having different amino-terminal parts. Although the function of the *c-ets-1* proteins is not known, it is tempting to speculate that they could have a common function, encoded by their common region (a to F), but directed towards different targets according to their specific N-terminal part encoded by the alternate 5' exons (α, β, or I⁵⁴). In that view, it is important to note the high level of proline residues of the α and β exons (10 residues of 71 [14%]), a criterion which has often been associated with nuclear proteins (58).

Although p54^{c-ets-1} has been described as a cytoplasmic protein (19), the chicken and mouse *c-ets-2* products have been localized in the nucleus (4, 7). Preliminary results indicate that in cell fractionation experiments of COS-1 cells transfected by the p68^{c-ets-1} cDNA clones, a small but significant proportion of p68^{c-ets-1} could be located in the nucleus (D. Leprince, unpublished results).

Splicing of *c-ets-1* exon a to upstream sequences can occur over long distances (ca. 40 kbp). In addition, splicing to exons α and β jumps exon I⁵⁴. This observation supports previous experiments in vivo (1) and in vitro (51) which argued against the progressive scanning mechanism for splicing. The mechanism by which the complete p54^{c-ets-1} and p68^{c-ets-1} mRNA species are generated is not clear. In particular, it remains to be determined how the 5' and 3' noncoding sequences of these RNAs relate to each other. Cloning and sequencing of longer cDNAs are required to resolve this point.

These experiments also address the differential expression of the *c-ets-1* proteins. The ubiquitous expression of p54^{c-ets-1} predominantly in thymic and bursal lymphocytes versus the expression of p68^{c-ets-1} found so far only in the spleen could result from a tissue-specific splicing of the same transcriptional unit, from the use of different promoters, from different 5' and 3' noncoding exons yielding two distinct but similar-sized mRNAs, or from a combination of these mechanisms. The restricted expression of p68^{c-ets-1} to the spleen, which is a peripheral lymphoid tissue, and the wide expression of p54^{c-ets-1} in the thymus and bursa of Fabricius, which are central lymphoid tissues, are characteristic of a protein family involved in various stages of the lymphoid maturation pathway. However, the ontogeny of the spleen is quite complex. Although its chief functions are the manufacture of leukocytes and the destruction of erythrocytes, during embryonic life the spleen is hemopoietic, with erythropoiesis and granulopoiesis both being very active (43). Thus, p68^{c-ets-1} could be expressed in a nonlymphoid cell type. The nature of the cell type(s) expressing

FIG. 5. Tissue-specific expression of $p68^{c-ets-1}$ mRNA. (A) Hybridization to a cDNA probe specific for $p68^{c-ets-1}$ (*EcoRI-PstI* 190-bp fragment) to the indicated chicken tissues and lymphoid cells lines. Autoradiography was for 3 days. (B) The same Northern (RNA) blot was stripped and rehybridized with a probe corresponding to the common exons (a to F) (*EcoRI-HpaI*; 1.2-kbp fragment). Autoradiography was for 1 day. (C) RNase protection experiments. The structure of the [32 P]cRNA probe and predicted fragments is shown diagrammatically at the bottom of the figure. Boxed portions of the restriction enzyme cleavage maps indicate cDNA sequences; single lines indicate plasmid sequences. Transcription orientation with the T7 promoter is also indicated. Restriction sites: B, *BamHI-BglII*; E, *EcoRI*; H, *HpaI*. Lanes: 1, probe only; 2, control tRNA coli (5 μ g); 3, total RNA from 2-day-old chicken thymus (20 μ g); 4, total RNA from 2-week-old chicken thymus (20 μ g); 5, total RNA from the T lymphoid cell line MSB1 (20 μ g); 6, total RNA from 2-day-old chicken spleen (20 μ g). Exposure was for 12 h for lanes 1 to 4 and 2 days for lanes 5 and 6. The extra bands in lane 6 represent full protection of degradation products of the probe (compared with lane 1; the bands are trimmed by 42 bp corresponding to the polylinker).

$p68^{c-ets-1}$ is currently being investigated by in situ hybridization and analyses of purified spleen cell populations.

Second, our results shed light on the mechanism of transduction of *c-ets*. In striking contrast with the other avian retroviruses carrying two *onc* genes (avian erythroblastosis virus and MH2) (10, 57), in E26 the two oncogenes are expressed in a single fusion protein of M_r 135,000 ($P135^{gag-myb-ets}$) (6, 23). We demonstrate that the two cell-derived oncogenes were apparently fused by an abnormal splicing mechanism between a cryptic splice donor site in the coding *myb* sequence (E6) and the normal splice acceptor site of the *c-ets-1* exon α (Fig. 6 and 7). Such aberrant splicing events have been previously reported for the acti-

vation of the mouse *c-myb* oncogene in Abelson virus-induced plasmacytoid lymphosarcomas (44) and for the chicken *c-erbB* activation in avian leukosis virus-induced erythroblastosis (38). Since a recombination between processed mRNA species occurring exactly at splice junctions seems unlikely, we suggest instead a mechanism based on one of the following models: (i) intermolecular splicing between RNA molecules (25, 50); (ii) a DNA translocation

c-DNA p68	 TA AGT AGC ACC TCA
<i>c-ets-1</i> exon α	 tct gca gGC ACC TCA
E26	 GAT CAT gGC ACC TCA
<i>c-myb</i> exon E6	 GAT CAT GGT TGC TTA
Splice donor consensus sequence	<div style="display: flex; align-items: center;"> <div style="text-align: center;"> AAGgt a.d </div> <div style="margin: 0 10px;"> aag t </div> <div style="text-align: center;"> 6 10 </div> </div>

FIG. 6. Genesis of the *myb-ets* sequence of E26 by aberrant splicing between a cryptic splice donor site in *c-myb* exon E6 and the normal splice acceptor site of *c-ets-1* exon α . The 5' point of *c-ets* transduction corresponds to the splice acceptor (s. a.) site of *c-ets-1* exon α . The 3' point of *c-myb* transduction, although located in a *c-myb*-coding exon, namely E6, (24), presents salient homology with a splice donor consensus sequence (36). The arrow in E26 indicates the accurate *myb-ets* boundary (the present study), which was previously assigned to the following G based on homology with the *c-myb* sequence (39, 40).

FIG. 7. Alternative splicing within the *c-ets-1* locus and genesis of the E26 *myb-ets* sequence. From top to bottom: schematic drawing of the *c-ets-1* locus organization and of the two *c-ets* cDNA types generated by alternative splicing within this locus. The E26 *myb-ets* sequence is generated through an abnormal splicing mechanism.

event between *c-myb*, *c-ets-1*, and a proviral DNA; and (iii) integration of a virus carrying only one of the oncogenes in the vicinity of the second cellular proto-oncogene.

Although we have no experimental evidence for the third model, it seems more favorable than the other models. Furthermore, an abnormal splicing event has been proven to be efficient in avian leukosis virus-induced erythroblastosis. In that particular situation, an avian leukosis virus integrates into the *c-erbB* gene and, via an aberrant splicing between *env* and *erbB* sequences, generates with a high frequency (about 50%) retroviruses carrying truncated *erbB* oncogenes (34, 38).

A second recombination between the hybrid RNA transcribed from this first recombinational event and the RNA genome of a helper virus could occur via "copy choice" during reverse transcription (5, 21, 56). During this last step, the reverse transcriptase could also generate the differences observed in the carboxy-terminal part of *v-ets* as compared with *c-ets-1* (15; Duterque-Coquillaud et al., in press) (Fig. 2). This alteration in its carboxy-terminal part represents, together with its linkage to the nuclear oncogene *myb*, the major differences observed between the coding regions of the cellular protein p68^{*c-ets-1*} and the *v-ets*-encoded part of the viral transforming protein P135^{*gag-myb-ets*}. Similar examples of cellular and viral proteins differing at their carboxy-terminal ends and the biological relevance of this alteration have been well documented (11, 12, 37, 54, 55). With the availability of our cDNA clones, we are currently constructing E26 mutants in which different parts of the *v-ets* oncogene, especially the 3' end, are replaced by corresponding parts of the cDNA to analyze the relevance of these mutations in the oncogenic spectrum of E26.

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