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Chromosomal reallocation of the chicken *c-myb* locus and organization of 3′-proximal coding exons

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In the course of our studies concerning the tissue-specific expression of the *c-myb* proto-oncogene, we have established the nucleotide sequence of the chicken *c-myb* 3′-proximal coding exons. In situ hybridization performed with different genomic DNA probes corresponding to nearly all the *c-myb* gene allowed us to localize the corresponding locus on the large acrocentric chromosome 3 in chicken. Our sequencing data also indicate that the 3′-proximal noncoding sequences represented in *c-myb* mRNA species are derived from non-contiguous exons.

c-myb proto-oncogene; Coding exon; Chromosome mapping; Avian genome; Lymphoma; Splicing

1. INTRODUCTION

The *c-myb* proto-oncogene is an evolutionarily well-conserved gene which has been identified in numerous vertebrates including chicken [1–5], human [1,6] and mouse [1,7]. In the past years, seven *c-myb* exons have been mapped in the chicken genome on the basis of their homology to the *v-myb* oncogene of Avian Myeloblastosis Virus (AMV) [2–5] and the use of viral probes permitted to assign the *c-myb* locus on a subset of large microchromosomes [8]. Similar studies performed in mouse have shown that the organization of the murine *c-myb* sequences homologous to the AMV oncogene is very close to that established in chicken [9] and have led to assign the mouse *c-myb* locus on chromosome 10 [10]. In humans, the organization of the *c-myb* sequences has been only performed by means of molecular hybridizations with *v-myb* or *c-myb* cDNA probes and the precise structure of the *c-myb* exons remains to be elucidated [11,12]. Use of genomic *c-myb* probes allowed mapping of the human *c-myb* locus on chromosome 6q23-25 [13–15].

It is now well-established that in all the species where it has been studied, the expression of the *c-myb* proto-oncogene occurs preferentially in immature hematopoietic cells [16–21]. There are also several pieces of evidence suggesting that the *c-myb* proto-oncogene plays a key role in the cellular proliferation and differentiation processes [22–25]. In a previous study [26], we have reported the characterization of the three 5′-proximal *c-myb* exons corresponding to the sequences identified at the 5′ end of *c-myb* mRNA species isolated from human, murine and chicken hematopoietic cells [12,27–29]. We have now established the organization of the 3′ coding exons in chicken. The isolation of specific probes representing most of the 5′- and 3′-proximal *c-myb* exons has allowed us to reallocate the *c-myb* locus on a large chromosome in chicken.

We also present evidence indicating that the 1.5 kb of non-coding sequences located at the 3′ end of *c-myb* mRNA species are generated by at least one splicing event.

2. MATERIALS AND METHODS

2.1. *Sources of DNA*

High-molecular-weight chicken DNA (>35.0 kb) was purified from C/O H & N 13-day-old chicken embryos by phenol extraction under the conditions previously described [30].

The λ533 recombinant cloned from the λ Charon 4A library of leukemic chicken DNA partially digested with *Eco*RI was already described [4]. This clone which contains the 3′ end of the *c-myb* sequences characterized thus far, was used as a source of DNA for sequencing and preparation of subclones. Clone P873 was already described [4] and also used as a source of DNA for sequencing.

2.2. *DNA blotting and hybridizations*

The preparation and labelling of DNA fragments used as probes in hybridization experiments were performed according to standard methods [30]. For Southern blot analysis, 10 μg of high-molecular-weight genomic DNA were digested with the appropriate restriction endonuclease, fractionated on 0.8% agarose gels and transferred on-

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to nitrocellulose [30]. A mixture of *Hin*dIII-digested λ DNA and *Hae*III-digested φX174 (RF) DNA was used as molecular weight markers. Prehybridization and hybridization procedures were already described [30].

2.3. *Derivation of the* c-myb *probes*

Two probes used in this study were derived from a 2.9 kb subset of a chicken thymic *c-myb* cDNA cloned in bluescript (kind gift of Drs Rosson and Reddy) [31]. These probes were obtained after digestion with *Xho*I and *Nco*I restriction endonucleases and used to map the *c-myb* 3′-proximal exons in chicken genomic DNA (Fig. 1).

Probe CNN3 contains most of the *c-myb* sequences transduced in AMV and 362 base pairs (bp) of downstream *c-myb* specific coding sequences (see Fig. 1). Probe CNX4 contains 230 bp of 3′-proximal *c-myb* coding sequences and 700 bp of downstream noncoding sequences. Probe CXX contains the whole 2.9 kb subset of the *c-myb* cDNA.

The isolation and characterization of clones P804, P542 and P873 have been described previously [4,26]. Clone P804 contains the *c-myb* sequences corresponding to the 5′-proximal terminus of the chicken lymphoma *c-myb* cDNA [26,29] and coding sequences specific to the normal thymic *c-myb* cDNA [26,31]. Clone P542 contains two *c-myb*-specific exons (E2 and E3 in Fig. 5) and two exons (E4 and E5 in Fig. 5) also represented in the *v-myb* oncogene of AMV [4,5]. Clone P873 was shown to contain the most 3′-proximal *c-myb* exon (E10 in Fig. 5) represented in this oncogene [4,5].

2.4. *DNA sequencing*

Dideoxy sequencing reactions were performed with α-^{35}S dATP, single-stranded M13 recombinant templates and Klenow (Boehringer Mannheim) or T7 DNA polymerase (Pharmacia) under the conditions previously described [30]. GC compressions were resolved by using the deaza sequencing kit from Pharmacia.

The M13mp18 and mp19 vectors were the recipients of the different fragments to be sequenced. As depicted in Fig. 2, we took advantage of rare restriction sites represented in both genomic DNA and thymic *c-myb* cDNA subclones to localize the *c-myb* 3′-proximal sequences and to generate M13 recombinant deletion mutants.

2.5. *In situ hybridization*

2.5.1. Preparation of chicken chromosomes

Secondary cultures of chicken embryo fibroblasts were established as already described [32]. To obtain metaphase chromosomes, MEM medium was replaced by TC 199 medium. Cells were treated for 90 min with colchicine, for 20 min with a hypotonic solution composed of 1:6 (v/v) human plasma:distilled water and finally fixed in 3:1 (v/v) ethanol:acetic acid overnight. Slides were kept at −20°C.

2.5.2. Hybridization and detection of the probes

The procedure previously described [33] can be summarized as follows: the probes were labelled with biotin-11-dUTP according to Gibco-BRL (Bethesda Research Laboratories) instructions. Slides were treated with RNase (Sigma, 100 μg/ml, 2 × SSC) for 1 h at 37°C, rinsed in 2 × SSC and dehydrated in 50%, 70% and 100% ethanol. Chromosomal DNA was denatured in 70% formamide, 2 × SSC at 70°C for 2 min. Slides were rinsed in 2 × SSC, dehydrated in ethanol and incubated overnight at 42°C with 15 μl of hybridization buffer containing 5–7 ng/ml of biotinylated probe. The probe was denatured for 5 min at 100°C in the same hybridization buffer composed of 50% formamide, 10% dextran sulfate, 2 × SSC, 40 mM sodium phosphate, 0.1% sodium dodecylsulfate, 1 × Denhardt's solution, pH 7.0.

Following the overnight incubation, slides were rinsed 2 times for 2 min in 50% formamide, 2 × SSC, 2 times for 2 min in 2 × SSC at 42°C and finally 5 min in a modified PBS (phosphate-buffered saline solution with 0.1% Tween 20 and 0.15% of bovine serum albumin added). Slides were then incubated for 1 h at 37°C with 50 μl of anti-biotin antibody (Vector Laboratories) diluted to 1:250 in modified PBS, rinsed 2 times in modified PBS and incubated for 1 h at 37°C with 50 μl of a fluorescein-conjugated IgG (Tebu-Nordic, France) diluted to 1:20 in modified PBS. For chromosome counterstaining, slides were incubated 5 min with propidium iodide at a final concentration of 1 μg/ml. After a brief PBS rinse, slides were mounted with PPD (*p*-phenylenediamine, 34) and photographed (Axiophot Zeiss, filter combination 487919).

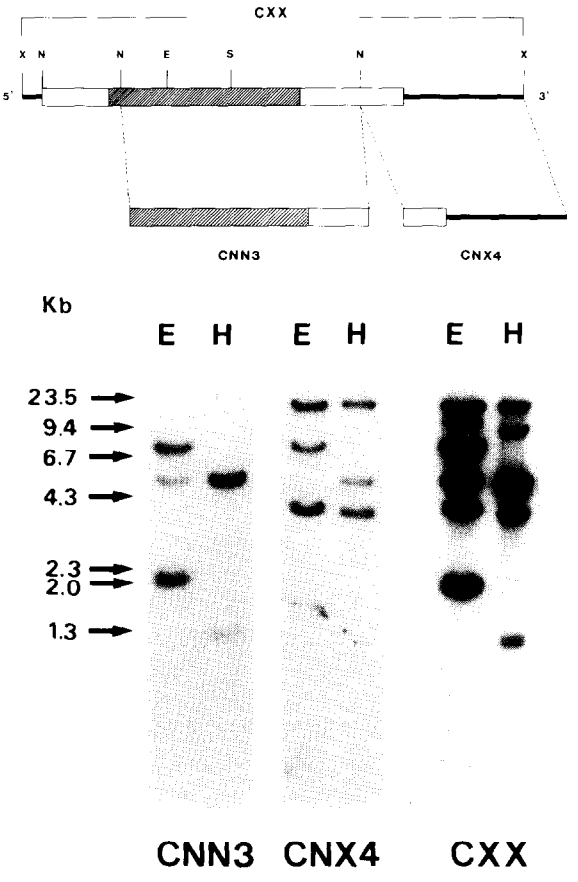

Fig. 1. Distribution of *c-myb* sequences among chicken DNA restriction fragments. (Top) Derivation of the probes. All probes were prepared from the thymic *c-myb* cDNA previously cloned in Bluescript (kind gift of Drs Rosson and Reddy) and designated CXX in this paper. The CNN3 and CNX4 fragments were obtained following digestion with *Xho*I (X) and *Nco*I (N) restriction endonucleases. Open boxes represent *c-myb*-specific sequences. Dashed boxes correspond to the *c-myb* sequences contained as *v-myb* in AMV. Solid bars correspond to 5′- and 3′-noncoding sequences. Restriction sites: E, *Eco*RI; N, *Nco*I; S, *Sal*I; X, *Xho*I. (Bottom) Southern blot analysis of chicken genomic DNA. High-molecular-weight DNA (>35.0 kb) was obtained from 13-day-old C/O embryos. Samples (10 μg per lane) were digested with *Eco*RI (E) or *Hin*dIII (H) restriction endonucleases, electrophoresed in 0.8% agarose gels, transferred onto nitrocellulose filters and hybridized with the indicated probes. A mixture of *Hin*dIII-digested λ DNA and *Hae*III-digested φX174 (RF) DNA was used as molecular weight markers.

3. RESULTS

3.1. *Characterization of DNA fragments containing the 3′-proximal* c-myb*-specific sequences in the chicken genome*

In normal C/O chicken DNA, the *c-myb* sequences homologous to the transforming gene of AMV are

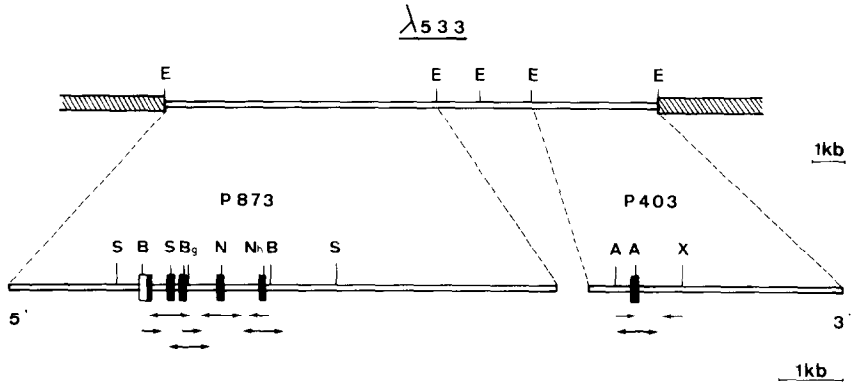

Fig. 2. Organization of 3'-proximal *c-myb* exons. The structure of the λ533 clone has already been described [4]. P403 and P873 contain the 4.0 and 8.7 kb *Eco*RI fragments subcloned in pUC18 and pBR322 vectors, respectively. The sequencing strategy is indicated by arrows. The open box represents the subset of *c-myb* sequences located at the 3' end of the *v-myb* oncogene in AMV. Filled boxes represent *c-myb*-specific exons. Restriction sites: A, *Ava*II; B, *Bal*I; Bg, *Bgl*II; E, *Eco*RI; N, *Nco*I; Nh, *Nhe*I; S, *Sac*I; X, *Xba*I.

distributed among 5 contiguous *Eco*RI fragments whose sizes are 5.4, 1.2, 2.1, 2.0 and 8.7 kb [3,4]. In order to characterize the 3'-proximal *c-myb* exons and establish the structure of this gene in chicken DNA, hybridizations of *Eco*RI- and *Hin*dIII-digested DNA were performed with the CNN3, CNX4 and CXX probes derived from the thymic *c-myb* cDNA (see Fig. 1 and section 2). Hybridization with the CNN3 probe led to the detection of DNA fragments whose sizes are in accordance with previously published data (8.7, 5.4, 2.1, 2.0 kb *Eco*RI fragments; 5.2 (double) and 1.2 kb *Hin*dIII fragments). Hybridizations performed with the CNX4 probe allowed us to detect three *Eco*RI DNA fragments (23.0, 8.7 and 4.0 kb) and three *Hin*dIII fragments (>25.0, 5.2 and 3.7 kb).

When the CXX probe was used as a control, all *myb*-containing DNA fragments were visualized. The relative intensities of hybridization for all DNA fragments were estimated by scanning the autoradiograms with an LKB Pharmacia Ultroscan and found to be in agreement with their content of *myb*-homologous sequences as subsequently determined by DNA sequencing (this work).

Hybridization experiments performed with the CNX4 probe and *Eco*RI-digested DNA from the λ533 recombinant phage also led to the detection of both 8.7 and 4.0 kb fragments (data not shown). These observations therefore indicated that at least two *c-myb*-specific exons were located downstream to exon E10 in the λ533 DNA.

3.2. *Characterization of the* c-myb *3'-proximal exons*

The strategy followed for sequencing is depicted in Fig. 2. The identification of the 3'-proximal *c-myb* exons could be established by comparison with the previously published sequences of different chicken *c-myb* cDNAs [29,31].

Four *c-myb*-specific exons were mapped in the 2.0 kb *Bal*I fragment of clone P873 (Figs 2 and 3). The 5'-proximal sequences of this fragment correspond to the end of the last *c-myb* exon (E10, see Fig. 5) represented in the AMV *v-myb* oncogene [4,5]. A 115 bp exon was localized at 400 bp downstream to the 5' end of this *Bal*I fragment, and 330 bp downstream to the 3' end of the last *c-myb* exon represented in *v-myb*. The 115 bp exon was separated from the next *c-myb* 126 bp exon by only 86 nucleotides, a distance which is very close to the minimal intron size of 66–81 nucleotides required for splicing to occur [35–37]. The 86-nucleotide intron was found to contain a pyrimidine-rich region (TTTCTTCCTTTC) upstream to its 3' end (AG), and at position −24 from its 3' boundary, a CTCAT motif which might correspond to an avian splice branch point (consensus for mammalians: Py U Pu A Py, [38]). Two other exons (111 and 108 bp) were identified in the 3' portion of the 2.0 kb fragment and their 5' boundaries localized respectively at 700 and 156 bp upstream from the 3' *Bal*I site. Both 5' and 3' exon/intron junctions of these exons correspond to the consensus splice acceptor and donor sequences (AG/ and /GT, respectively).

Fig. 3. Nucleotide sequence of chicken 3'-proximal *c-myb* exons. The genomic sequence (I) established in this work has been aligned with that of thymic cDNA (II) [31]. In order to indicate the 3' boundary of the fifth *c-myb* exon (designated by E15) characterized in this study, 206 bp of 3'-proximal noncoding sequences have been identified and added to the previously published [31] thymic cDNA sequence (see section 3). Mismatches between the two sequences are indicated by asterisks. Coding and noncoding sequences are represented in bold and italics, respectively. For the sake of clarity, only 20 nucleotides of intronic sequences (plain text) are indicated upstream and downstream of each exon, except for the 86-nucleotide intron separating exons E11 and E12. The 3'-proximal *c-myb* exons are numbered according to the nomenclature defined in the text. The end of homology between exon E10 and the AMV *v-myb* oncogene is indicated by a vertical arrow. The translation stop-codon identified in both genomic and cDNA sequences is underlined.


E10
E11
E11
E12
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E15

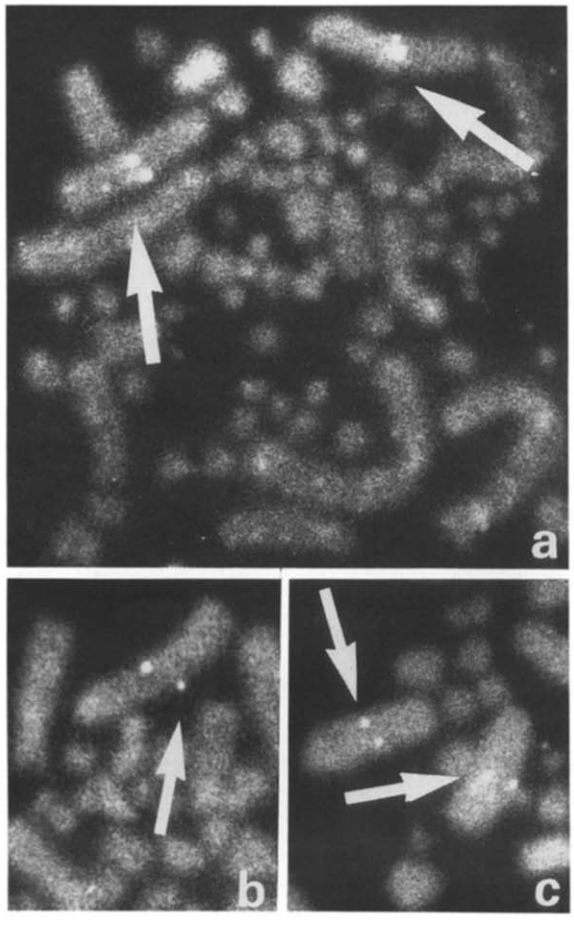

Fig. 4. In situ hybridization of biotinylated probes. In situ hybridization on prometaphase or metaphase chromosomes from chicken fibroblast cultures. (a) Probe P804, (b) probe p542 and (c) probe p873. For these three probes, recurrent fluorescent spots were observed on chromosome 3. Immunofluorescein detection and propidium iodide counterstaining.

A fifth *c-myb*-specific exon was localized at 700 bp from the 5′ end of the 4.0 kb *Eco*RI fragment derived from λ533 and subcloned as P403 (see Figs 2 and 3). This exon contains 117 bp corresponding to the 3′-terminal coding sequences represented in chicken *c-myb* cDNAs isolated from either lymphoma [29] or normal thymic cells [31]. Downstream to the translation stop codon we could also recognize the stretch of 3′ noncoding sequences previously identified [31]. As mentioned above, hybridization experiments performed with the CNX4 probe indicated that the *c-myb* sequences contained in this probe were distributed into three *Eco*RI fragments (8.7, 4.0 and 23.0 kb, see Fig. 1). Since the last *c-myb* coding exon was localized in the 5′-proximal part of the 4.0 kb *Eco*RI fragment (see Fig. 2), the noncoding sequences contained in the CNX4 probe were likely to be split in at least two exons. Indeed, comparison of the nucleotidic sequences that we established from both the genomic DNA and the 3′-proximal noncoding region of the thymic *c-myb* cDNA allowed us to confirm that only 233 bp of noncoding sequences were contained in the last *c-myb* exon identified in this work (Fig. 3). Downstream to the 3′ end of this exon, sequences contained in genomic DNA and cDNA were found to diverge (see Fig. 3), indicating that at least one splicing event is required to generate the 1500 bp of noncoding sequences located at the 3′ end of *c-myb* mRNA species. The nucleotidic sequences established from both genomic DNA and *c-myb* cDNA did not allow us to identify a splice donor consensus signal at the 3′-proximal end of this fifth exon.

3.3. *Chromosomal reallocation of the* c-myb *locus*

An attempt to localize the *c-myb* proto-oncogene on the chicken genome was performed a few years ago by means of in situ hybridizations with Avian Myeloblastosis Virus RNA probes [8]. The genomic probes constructed in our laboratory and which contained either the 5′-proximal *c-myb*-specific exons characterized thus far [26] or the 3′-proximal *c-myb*-specific exons (this work) allowed us to reexamine the chromosomal localization of the chicken *c-myb* locus.

Following in situ hybridization with the P804, P542 or P873 probe, fluorescent spots were recurrently observed in the middle segment of the long arm of a large acrocentric chromosome (Fig. 4), which corresponds to chromosome 3, in the classification of Ohno [39]. In many metaphases, double spots existed, one on each sister chromatid. The proportion of metaphases exhibiting at least one spot on chromosome 3 ranged from 60 to 80% depending on the probe used and on its concentration during the hybridization procedure. Very few spots were observed randomly on other chromosomes.

4. DISCUSSION

To date, the *c-myb* sequences represented in all cDNAs isolated from human, murine and chicken cells have not yet been completely identified at the genomic level. In a previous work [26] we have characterized three *c-myb*-specific exons (E1 to E3 in Fig. 5) whose sequences correspond to the 5′-part of the *c-myb* cDNA isolated from chicken lymphoma cells [29]. These sequences are also homologous to the 5′-proximal region of the human and murine *c-myb* cDNAs isolated thus far [12,27,28]. In this paper we present the genomic organization of the 3′-proximal chicken *c-myb* sequences which are expressed in both chicken lymphoma and normal thymic cells [29,31]. We have identified five additional *c-myb*-specific exons containing all the coding sequences represented at the 3′ end of both lymphoma and thymic cDNAs. Since the *c-myb* sequences homologous to the *v-myb* oncogene of AMV were found to be split into seven exons [2–5], our present results and previous ones [26]

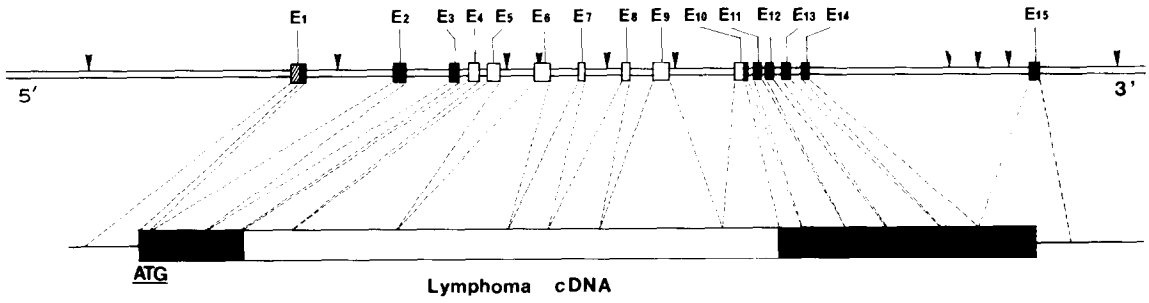

Fig. 5. Organization of the chicken *c-myb* proto-oncogene. Open boxes denote the *c-myb* sequences represented in the AMV *v-myb* oncogene, while filled boxes represent *c-myb*-specific exons. All *c-myb* exons are numbered according to the nomenclature defined in the text. Exon E1 corresponds to the most 5'-proximal sequences of the *c-myb* locus mapped on chromosome 3 (see sections 3 and 4). The dashed part of exon E1 designates sequences which are noncoding in lymphoma cDNA and coding in thymic cDNA. Arrowheads on the genomic map indicate the positions of *Eco*RI restriction sites. The position of the ATG codon identified in the lymphoma cDNA is indicated.

therefore indicate that the *c-myb* coding sequences represented in the lymphoma cDNA are distributed into fifteen exons spanning over 35.0 kb of chicken genomic DNA (Fig. 5).

In thymocytes, *c-myb* RNA species contain additional 5'-proximal coding sequences [31] which are not represented in the *c-myb* transcripts expressed in other hematopoietic tissues ([40], and our unpublished observations). Since our results show that the bulk of fifteen exons characterized thus far is common to both B and T cell *c-myb* mRNA species, we have proposed to designate the first exon E1 (Fig. 5), each other upstream exon expressed in a particular cell lineage being designated by a letter indicating the origin of the tissue.

The results reported in this paper suggest that splicing of 3' noncoding sequences could play a role in the differential expression of the *c-myb* proto-oncogene. Although we do not know at the moment if the *c-myb* 3'-proximal noncoding sequences contain an A/U-rich stretch [41] similar to that involved in the decreased stability of *c-fos* mRNA species [42], splicing of these noncoding sequences could be involved in the differential stability of *c-myb* mRNA species observed during differentiation [23] and progression throughout the cell cycle [21].

The isolation of recombinant genomic DNA clones containing the fifteen *c-myb* coding exons ([3,4,26] and this work) allowed us to perform in situ hybridizations and show that the chicken *c-myb* locus is localized on the large chromosome 3 (see Fig. 4). Our results are in disagreement with previous mapping of the chicken *c-myb* gene on a subset of large microchromosomes [8]. This discrepancy is most likely due to the different sources of probes used. While we used cloned *c-myb*-specific probes covering 22.0 kb of genomic sequences, Tereba and Lai [8] employed purified AMV genomic RNA which only contains a subset (1.1 kb) of spliced *c-myb* sequences [4,5,31,43,44]. The locus mapped by Tereba and Lai might therefore correspond to viral-specific sequences instead of *c-myb*-related sequences. The localization of the *c-myb* proto-oncogene on chromosome 3 argues against the nonrandom distribution of cellular oncogenes on microchromosomes and allows the reexamination of whether exogenous retrovirus genomes preferentially integrate near the *c-myb* gene.

To date, the oncogenic activation of the *c-myb* proto-oncogene has been associated, in chicken, with retroviral insertions in the 5'-proximal *c-myb* sequences [45,46]. The characterization of the *c-myb* exons described in this paper will now enable us to determine whether viral-induced disruptions of the 3'-proximal *c-myb* locus are, as already reported in mouse [9,47–49], also involved in the generation of avian hematopoietic neoplasms.

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