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Multiple Transcription Start Sites and 5' Alternate Splicing of Murine IL-3 Receptor β-Chain Transcripts

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Summary: The murine interleukin-3 receptor β-chain genes, IL-3Rβ_{IL-3} and IL-3Rβ_c, encode the signal transducing chains of the high affinity receptors for IL-3 and IL-3, GM-CSF and IL-5 respectively. Little is known about the regulation of their expression. To enable the study of the promotors of IL-3Rβ_{IL-3} and IL-3Rβ_c, we have characterized their respective 5' untranslated regions using a modified 5' RACE protocol. Four classes of alternatively spliced transcripts were isolated that initiate in a 400 nt region upstream from a previously reported start site(1). The initially reported partial IL-3Rβ_{IL-3} clone belongs to the first class of transcripts(2). The second class starts in the middle of an intron as defined by the first class. The 3rd and 4th class establish 2 novel splice donor sites. These results were confirmed by RNAse-protection assay. The complex organization as evident from our data establishes an experimental framework for future experiments aimed at the study of the promoters for the murine IL-3Rβ genes.

The mouse genome has two highly homologous genes , IL-3Rβ_{IL3} and IL-3Rβ_c, that code for the β-subunits of the IL-3 receptor and the IL-3, GM-CSF and IL-5 receptors respectively. IL-3Rβ_{IL3} (or AIC2A) is unique among the known β-chains in that it binds IL-3 on its own, with low affinity(3). It selectively associates with the IL-3Rα-chain to make a high affinity receptor. Unlike IL-3Rβ_{IL3}, IL-3Rβ_c does not bind IL-3, but it associates with the α-chains of the IL-3, IL-5 and GM-CSF receptors to form functionally competent high affinity receptors(2). IL-3Rβ_c (or AIC2B) is the functional equivalent of the human KH97 gene (or β_{common})(4). IL-3Rβ_{IL3} and IL-3Rβ_c are highly homologous (91% conservation at the amino acid level) and are closely linked on chromosome 11(1).

To enable the study of the regulation of the expression of the muIL-3Rβ–chains, it is essential to carefully map the transcription initiation sites for both genes. Primer extension of IL-3Rβ_{IL3} and IL-3Rβ_c transcripts mapped a start site to a region which is highly conserved between the two genes and shows some sequence conservation with the transcription initiation sites of the promoters of the EPO and IL-7 receptors (1). However, the isolation of an IL-3Rβ_c cDNA corresponding to a transcript originating upstream of the identified start site (1-2) implied the

Abbreviations:
EPO(R): erythropoietin (receptor); GM-CSF: granulocyte-macrophage colony stimulating factor; IL-3: interleukin 3; IL-5: interleukin 5; IL-7: interleukin 7; nt: nucleotides; RACE: rapid amplification of cDNA ends; S.A.: splice acceptor site; S.D.: splice donor site.

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existence of additional start sites, at least for this gene. Unfortunately, the position of these sites remained unknown and it was unclear whether similar transcripts also existed for IL-3Rβ_{IL3}. Little is known about the mechanisms regulating IL-3R expression, although the expression of the two β–genes appears to be linked (5). Preliminary experiments linking various fragments starting upstream of the previously mapped start site to the reporter gene human growth hormone revealed weak promoter activity associated with this region. Although the activity was preferentially observed in hematopoietic cells, it was not strictly restricted to these cells as basal activity was also measured in fibroblasts (B.M.-P., unpublished). More importantly, the putative promoters failed to upregulate expression of the reporter gene in response to hematopoietic growth factors, in contrast to what is observed for IL-3Rβ transcripts under the same conditions (6).

Therefore, we set out to characterize the IL-3Rβ_{IL3} and IL-3Rβ_c transcripts that originated upstream of the known initiation site and to map their start sites with the eventual goal of studying the promoter region around these new sites. Using a modified 5'-RACE method, we show that murine IL-3Rβ genes are characterized by a complex array of start sites spanning a region of about 400 nt and exhibit extensive alternative splicing in their 5' untranslated region.

Materials and Methods

Cell lines: The murine IL-3 dependent hematopoietic progenitor cell lines B6SutA (7), Ba/F3 (8), FDC-P1(9) and 32D (10) cells were maintained in RPMI 1640, supplemented with 10% fetal calf serum (FCS), 0.5 ng/ml of recombinant murine IL-3 (R&D Systems, Minneapolis, MN) and antibiotics (penicillin [50 units/ml] and streptomycin [50 μg/ml]). MKVS cells are early myeloid cells derived after infection of mouse bone marrow with a v-*src*/v-*myc* retrovirus and are factor independent for growth (B.M.-P, unpublished results). MKVS cells were maintained in RPMI 1640 complete medium in the absence of IL-3. All cells were kept in a 5% CO_2/95% air incubator at 37° C.

RNA isolation and polyA$^+$ mRNA selection. Total RNA was isolated by the guanidium thiocyanate/cesium chloride method as described (11). PolyA$^+$-mRNA was obtained from B6SutA total RNA by two rounds of selection over an oligo-dT cellulose column as described (12).

Primer extension assay. Total RNA (20 μg) or polyA$^+$ mRNA (2 μg) from the indicated cell lines was annealed overnight as described (12) to 5 ng of ^{32}P-5' labeled primer P1 (5'-CTGTATTGGTGCTTGGCTGGGGGTTTGGCTCCACTCATCTTACAG-3'). Primer P1 is complementary to both mouse IL-3Rβ mRNAs (IL-3Rβ_{IL3} and IL-3Rβ_c). Reverse transcription of the annealed products was achieved with Superscript II RNase H$^-$ MuLV reverse transcriptase (GIBCO BRL, Life Technologies, Gaithersburg, MD) according to the manufacturer's recommendation. After phenol extraction and ethanol precipitation, the reaction products were run on a 6.5% denaturing urea/polyacrylamide gel.

Modified 5'-RACE protocol . This modified method takes advantage of the property of T4 RNA ligase which can ligate single stranded DNA molecules in the presence of hexamine cobalt chloride (13). The modified 5' RACE reaction was performed with a kit (CLONETECH Laboratories, Palo Alto, CA) according to the manufacturer's instructions with minor modifications. 2 μg of polyA$^+$ B6SutA mRNA were annealed to primer P1 and reverse transcribed with AMV reverse transcriptase. DNA amplification was performed either with Pfu DNA polymerase (STRATAGENE, La Jolla CA) or with Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN). Primers used for amplification were the 5'-AmpliFINDER anchor primer provided in the kit (CLONETECH), and the 3' primer (P2) capAIC, an IL-3Rβ_{IL3} specific oligonucleotide (5'-GGGGGATCC**GCCAGTGAGCTGCAGGCT**-3'). The sequence homologous to IL-3Rβ_{IL3} in capAIC is shown in bold, and the underlined nt indicates the mismatch with IL-3Rβ_c. capAIC was designed to hybridize just upstream of the previously mapped major start site for IL-3Rβ (1). Amplification was carried for 35 cycles at the following parameters: Denaturation: 94°C, 45 sec.; Annealing: 55°C, 45 sec.; Extension: 72°C, 2 min. The PCR products were phenol-extracted and digested with Bam H1 (site included in the P2 primer) and Eco R1 (part of the AmpliFINDER anchor primer), before they were separated on a 10% polyacrylamide gel.

Isolated DNA products were subcloned into the Bam H1 and Eco R1 sites of pUC13. DNA sequencing was performed with Sequenase (USB, Cleveland, OH) according to the manufacturer's instructions.
RNAse protection assay. Probes: *BS-PAC*: The probe was obtained by PCR using a genomic clone of IL-3Rβ_{IL3} as DNA template and PAC-1 and PAC-2 primers. The 5' PAC-1 primer which includes a Bam H1 cloning site, corresponds to nt -70 to -53 of AIC2A (IL-3Rβ_{IL3}) sequence published by Gorman *et al* (1) , and the 3'primer PAC-2 corresponds to nt +62 to +80 of AIC2A. The PCR product was digested with Dde I, and the Dde I end was filled in with the large fragment of DNA polymerase I. The reaction product was then digested with Bam H1, and subcloned into the pBluescript II KS phagemid (STRATAGENE) between the Eco RV and Bam H1 sites. *a-T10*: The corresponding PCR product was cloned into the pBluescript II KS phagemid between the Eco R1 and Bam H1 sites. The BS-PAC and a-T10 antisense probe were generated using T3 and T7 RNA polymerase respectively. RNase protection assay was performed as described using 20 µg of total RNA (5).

Results and Discussion

Primer extension assay confirms the existence of several transcription initiation sites: To confirm the existence of multiple start sites for the β-chain transcripts, primer extension analysis was performed with various RNAs isolated from a number of murine hematopoietic cell lines and from a fibroblast cell line, NIH 3T3. Three prominent bands, ranging in size from 115 nt to 129 nt , are detected when RNAs isolated from hematopoietic cells are annealed to the P1 primer and reverse-transcribed (Fig. 1, lanes 3-6). The three bands are specific since they are not detected when tRNA or RNA from NIH 3T3 cells (which do not express the IL-3Rβ-chain genes) are similarly primed with P1 (Fig. 1, lanes 1-2). The size of the 129 nt fragment is in complete agreement with the assignment of the previously described start site for IL-3Rβ_{IL3} and IL-3Rβ_c (1). The two smaller bands do not result from premature termination, but represent *bona fide* start sites (see Fig. 2A). In addition to the strong triplet, three fainter bands, which migrate as 215, 240 and 275 nt species are observed with B6SutA polyA$^+$ RNA (Fig. 1, lane 6). Although the corresponding bands are barely visible in FDC-P1, B6SutA and Ba/F3 RNA (lanes 3-5), a longer exposure of the same gel confirms their presence. As expected, these bands are absent in tRNA and NIH3T3 RNA (lanes 1-2) even after longer exposure (Fig. 1, and data not shown). Thus, the bulk of the extension products for the IL-3Rβ_{IL3} and IL-3Rβ_c genes corresponds to transcripts originating from a cluster of three closely spaced sites at or near the previously reported site (1). In addition, there are less abundant products that extend between 100 and 160 nt further upstream of this start site.
RNAse protection assay detects upstream IL-3Rβ transcripts: The location of the start sites corresponding to the prominent bands in Fig. 1. is unusual in that it resides in the middle of exon 1 (Fig. 2B) present in an IL-3Rβ_c cDNA isolated by Gorman *et al.* (2). As primer extension experiments can be subject to artifacts caused by the presence of potential strong stops and resulting in premature termination of the reverse transcription reaction, we sought to confirm our results by RNase protection with a probe spanning the region of interest (Fig. 2B). RNA from hematopoietic cells specifically protected several bands (Fig. 2A). In agreement with the primer extension analysis, a cluster of three prominent bands was observed migrating at around 45 nt. These protected fragments originate from the start sites located in the middle of exon 1. A second class of protected fragment (migrating at 86 nt) corresponds to the predicted size of exon 1 (see

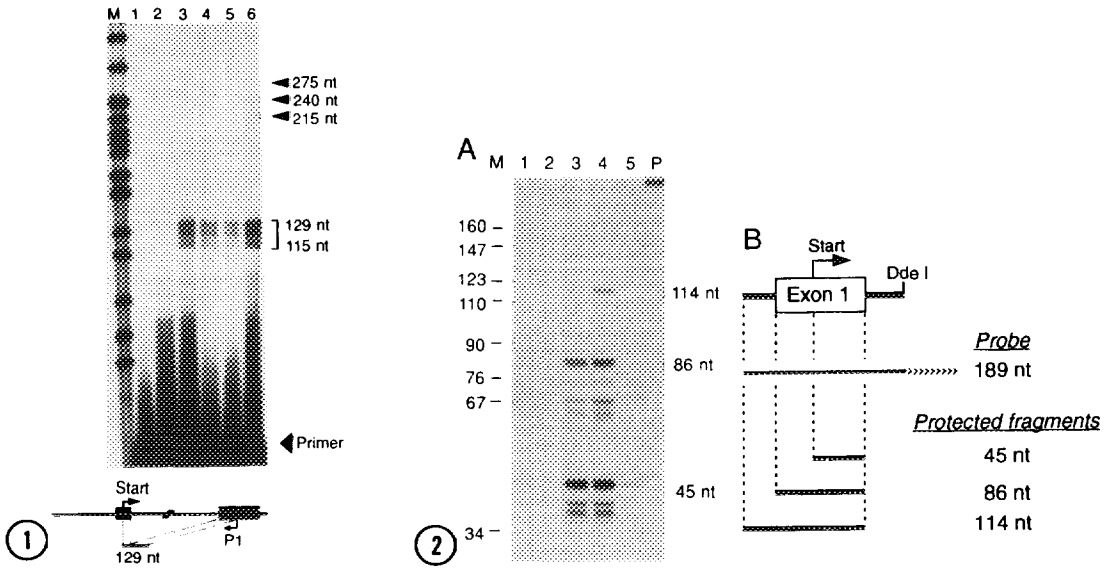

Figure 1. Primer extension of IL-3Rβ mRNAs.

Total RNA (20 μg) or polyA$^+$ RNA (2 μg) was reverse transcribed after hybridization with primer P1 as described in Material and Methods. Extension products were separated on a 6.5% denaturing urea/polyacrylamide gel. M: pBR322 Msp 1 fragments. 1: tRNA; 2: NIH 3T3 RNA; 3: FDC-P1 RNA; 4: B6SutA RNA; 5: Ba/F3 RNA; 6: B6SutA polyA$^+$ RNA. The length of the various products is indicated. Products originating at the previously mapped start site are indicated by the open bracket; filled triangles highlight specific products originating at new 5' start sites. Shown below is a diagram of the position of primer P1 relative to the IL-3Rβ gene and the expected length for the product corresponding to the previously mapped site(1).

Figure 2. RNase protection confirming the presence of start sites within exon 1.

A. Total RNA (20 μg) was hybridized to the BS-PAC probe described in Materials and Methods, and is diagrammed in panel B. M: pBR322 Msp 1 fragments. 1: tRNA; 2: NIH 3T3 RNA; 3: FDC-P1 RNA; 4: B6SutA RNA; 5: MKVS RNA; P: Undigested BS-PAC probe. B. The region around exon 1 of IL-3Rβ_{IL3} corresponding to the BS-PAC probe is shown. The nature of the specific protected bands corresponding to panel A is also depicted.

Fig. 3). Finally, the size of the slowest migrating protected fragment (114 nt) matches the length of the first exon plus the upstream IL-3Rβ_{IL3} specific sequence present in the probe, providing evidence for the existence of unspliced transcripts originating upstream of exon 1. Interestingly, the relative abundance of these transcripts seems to vary in different hematopoietic cells: these transcripts are easily detected in the hematopoietic progenitor cell lines B6SutA and FDC-P1 cells, but they are nearly absent in the more mature myeloid cell line MKVS (Fig. 2, compare lanes 3, 4 and 5). Other minor bands detected in Fig. 2A can be attributed to the presence of two single point mutations (11 and 32 nt respectively from the start of exon 1) between IL-3Rβ_{IL3} and IL-3Rβ_c in the area spanned by the IL-3Rβ_{IL3} specific probe.

Isolation of 5'-RACE IL-3Rβ clones: To further investigate the nature of the upstream transcripts, 5'RACE of B6Sut A cDNA primed with P1 was performed using a universal anchor primer (which had been ligated to the 5' end of the cDNA with T4 ligase) and a nested primer (capAIC) which hybridized just upstream of the previously mapped start site. The resulting PCR products yielded several discrete bands ranging in size from 160 nt to 180 nt on a non-denaturing

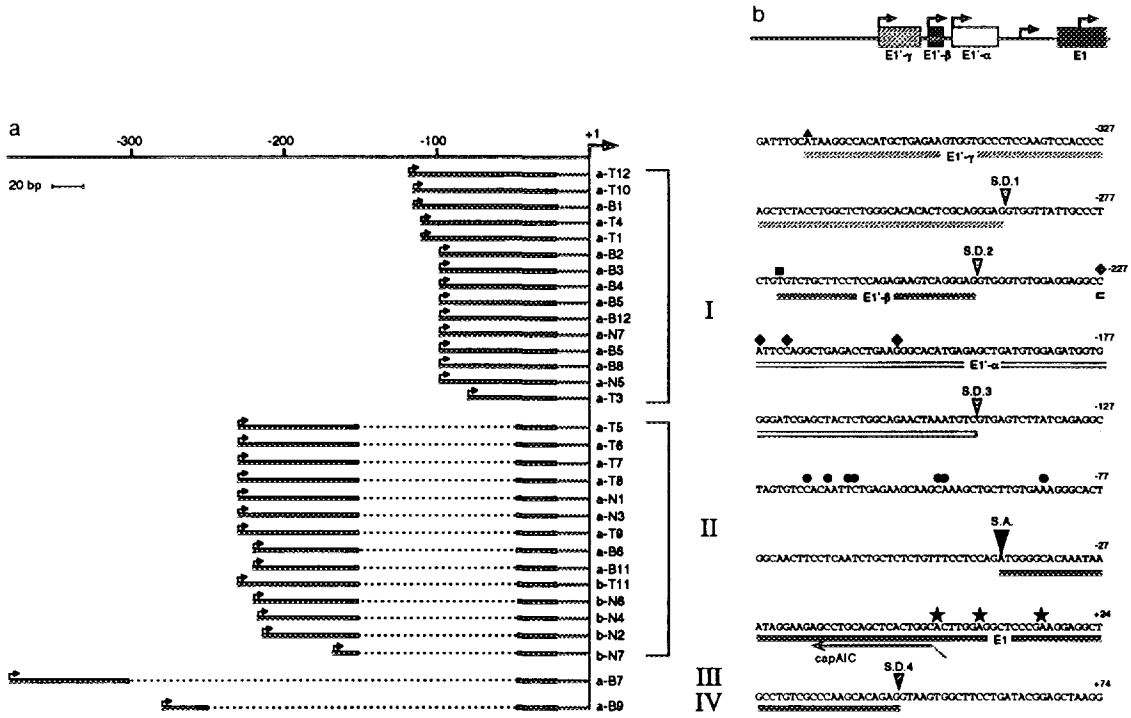

Figure 3. Isolation of 5' RACE clones corresponding to the new upstream start sites.
a. The structure (thick black line) and start sites for the 5' RACE clones are schematically indicated. +1 corresponds to the previously mapped start site(1). The thin gray box (▭) corresponds to the capAIC primer and the dotted line (·····) indicates intronic sequences. Clones derived from IL-3Rβ_{IL3} are indicated with the prefix "a", and clones derived from IL-3Rβ_c with the prefix "b". T, B and N are independent PCR reactions where Pfu polymerase was used in the first two and Taq was used in the last one. **b.** The position of new 5' exons for IL-3Rβ_{IL3} gene (boxes) and of all the start sites (arrows) are depicted on the top. Transcripts defining exons E1'-γ, E1'-β and E1'-α have been isolated. Shown below is the IL-3Rβ_{IL3} sequence from nt +74 to -376 and the position of the start sites corresponding to the different transcripts isolated by 5' RACE. Symbols (▲), (■), (◆), (●) indicate the different classes of 5' RACE clones isolated and are positioned above the start of each upstream transcript. Symbol (★) is positioned above the start sites corresponding to the IL-3Rβ_{IL3} transcripts originating within exon 1 (see Fig.1). Nucleotides corresponding to exons E1, E1'-γ, E1'-β and E1'-α are underlined with a thick line of the corresponding fill pattern of the exon boxes shown on the top of the diagram. The location of primer capAIC and the positions for S.D.1-4 and S.A. are indicated.

polyacrylamide gel (data not shown). Each band was isolated and subcloned into the pUC13 vector. Of 32 clones sequenced, 31 were derived from one or the other IL-3Rβ-chain gene (Fig. 3). A majority (26 out of 31) arose from IL-3Rβ_{IL3}. This bias is likely due to the choice of the nested primer rather than a reflection of the relative expression of the two genes in B6SutA cells. Indeed, the nested primer P2 (capAIC) is completely homologous to IL-3Rβ_{IL3} but contains one mismatch with the corresponding region in IL-3Rβ_c.

The isolated clones can be divided into 4 groups (Fig. 3). Roughly half of the clones initiate upstream of exon 1 in the middle of an intron. The existence of such transcripts was predicted by the previous RNase protection assays (114 nt band in Fig 2A). As in the transcripts originating within exon 1, there is evidence for staggered start sites. This was confirmed by an

RNase protection analysis using the insert in clone "a-T10" as a probe and RNAs from two other hematopoietic cell lines, FDC-P1 and Ba/F3. The two RNAs protected several bands ranging in size from 116 to 100 nt (data not shown). These bands are specific as they are not observed in cells lacking an IL-3R. The second class of transcripts shows the same 5' organization as the original AIC2B (IL-3Rβ_c) cDNA isolated by Gorman *et al.* (2). Since all of the 5'-RACE clones corresponding to IL-3Rβ_c belong to this group, it is likely that it is the most abundant form of upstream transcripts for this gene. As in the previous two groups, there is evidence for more than one initiation site. The last two classes of transcripts identify two new splice donor sites defined by two additional exons (clones a-B7 and a-B9). The splice donor sites utilized by these clones were predicted when the IL-3Rβ_{IL3} genomic sequence was analyzed by the GeneId program (14). They show a highly significant index of predictability for potential splice donor sites (0.85 and 0.73 respectively). Because only one clone for each form was isolated, we sought to confirm the existence of the corresponding transcripts by RNase protection. In both cases, bands of the predicted size were specifically protected by RNAs isolated from B6SutA, FDC-P1 and Ba/F3 cells but not by RNA from NIH 3T3 cells (data not shown).

Concomitantly with the characterization of the new transcripts, genomic clones for murine IL-3Rβ_{IL3} and IL-3Rβ_c were isolated. Sequence comparison of the two genes confirmed that the high degree of homology extends over a region 5' of the previously mapped start site. The major difference is the insertion of a 640 bp mouse repeat at position -745 in IL-3Rβ_c. The significance of this insertion is unclear, but as the two genes are coordinately expressed, it is unlikely to play an important role in the regulation of expression.

How multiple initiation sites and alternate splicing in the 5' untranslated regions of the IL-3Rβ genes contribute to their overall expression in hematopoietic cells is unknown. The multiple start sites may reflect the use of different promoters exhibiting differentially regulated activity in distinct hematopoietic cell lineages or during the course of differentiation. Indeed, we have found that the relative abundance of the various transcripts changes in hematopoietic cells of different lineage and/or differentiation state. The complex organization as evident from our data establishes an experimental framework for future experiments aimed at the study of the promoters for the murine IL-3Rβ genes. The latter is a critical first step in the process of deciphering the function of IL-3 and its receptor in hematopoiesis and the mechanisms involved.

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