ELSEVIER

Gene 219 (1998) 19–24

GENE
AN INTERNATIONAL JOURNAL ON GENES AND GENOMES

The region 3′ to Cα1 gene of human IG heavy chain displays a polymorphic duplicated sequence and encodes an RNA associated with polysomes

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Received 18 May 1998; received in revised form 21 July 1998; accepted 22 July 1998; Received by A. Sippel

Abstract

A highly spread polymorphism flanking the 3· Cα1 human IG heavy chain gene was identified. This polymorphism allowed the detection of an internal duplication within the 3′ flanking region of both Cα1 and Cα2. This region has a regulatory function with four enhancer structures also present at the 3′ end of the human Cα2 as well as in that of mouse and rat single Cα genes. The 5682-bp sequence of clone λpl8 described here starts 3′ of Cα1 and presents three open reading frames; one of them contains part of the tandem repeats with the 20-bp consensus described previously that is expressed in a poly(A)$^+$ RNA and found in three dbEST clones of the human tonsillar cDNA library. Here, we demonstrate that in the CLF1 B lymphoblastoid cell line, this transcript is associated with polysomes. We also discuss the possibility of the presence of a new regulatory gene that does not encode an immunoglobulin and maps in the human IG heavy chain gene cluster.

Keywords: 20-bp tandem repeats; IG regulatory region; Cα1–Cα2 duplication; GA repeats

1. Introduction

The human immunoglobulins (IG) constant (C) heavy chain cluster has a duplication of four genes (Flanagan and Rabbitts, 1982; Hofker et al., 1989): the more upstream γ3, γ1, ψϵ, α1 block is separated by ψγ from the more downstream γ2, γ4, ϵ, α2 block (Fig. 1A). The sequence, spanning about 30 kb at the 3′ end of Cα1, is repeated downstream of Cα2 because of the entire duplication of the region containing the four constant genes. It is possible that this region has a regulatory function with four enhancers recently found to form a locus control region (LCR) (Mills et al., 1997). These enhancers have been tested for their DNase I hypersensitivity and for expression of reporter genes in several lymphoid cell lines (Chen and Birshtein, 1997; Pinaud et al., 1997). This human LCR presents a high homology with murine Cα 3′ LCR (Chen and Birshtein, 1997). Several polymorphisms have been identified in the heavy chain constant region (Brusco et al., 1995, 1997); in particular, in the 3′ Cα1 region, we describe a polymorphism and a duplication also present 3′ of Cα2, as reported by others (Pinaud et al., 1997).

CpG islands have been identified in the large interval between Cα1 and ψγ (Sadhu et al., 1997), suggesting the possibility of the presence of new genes unrelated to immunoglobulines. In a previous study of a 5-kb sequence downstream of Cα1 gene, within the 18.5-kb long *Xba*I clone λpl8 (Fig. 1B), we have found that a sequence, which includes about 80 tandem repeats of a 20-bp consensus, is transcribed as polyadenylated RNA in several lymphoblastoid cell lines and in PBMC (peripheral blood mononucleated cells) (Gualandi et al., 1995). This RNA is unrelated to other IGH gene

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Abbreviations: C, constant; dbEST, database expressed sequence tags; HSE, heat-shock element; IGH, immunoglobulin heavy chain; LCR, locus control region; NCBI, National Center of Biotechnology Information; ORF, open reading frame; PBMC, peripheral blood mononucleated cells; RT-PCR, reverse trascriptase polymerase chain reaction.

0378-1119/98/$19.00
PII: S0378-1119(98)00399-0

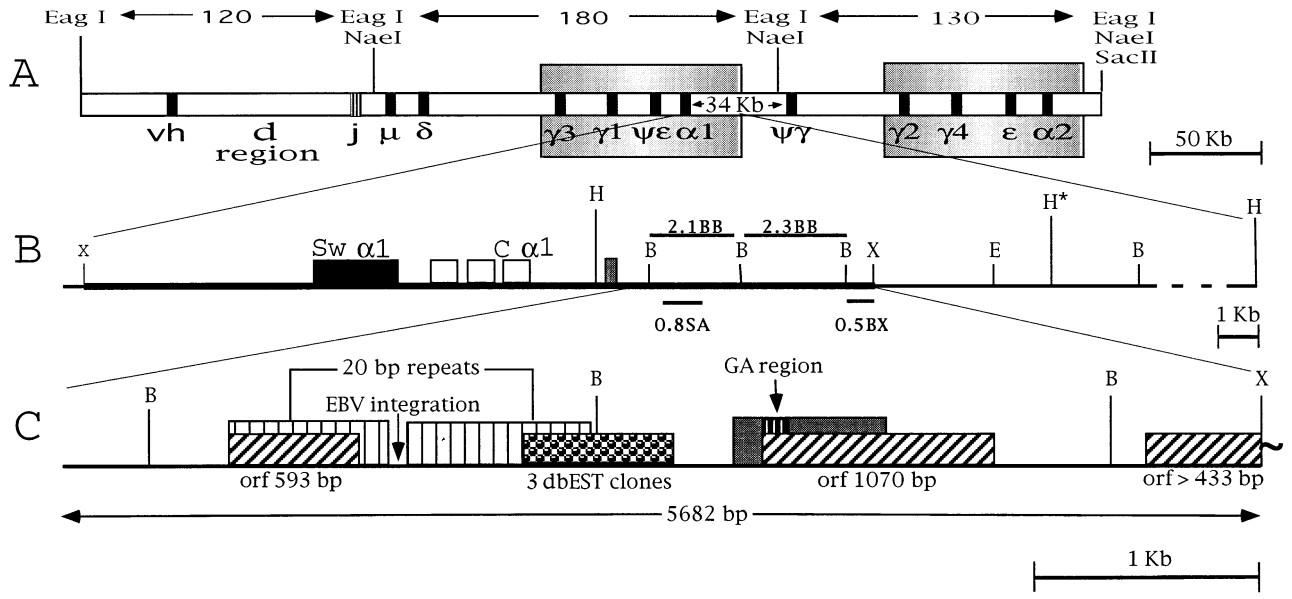

Fig. 1. (**A**) Human immunoglobulin heavy chain cluster (Hofker et al., 1989). The shadowed rectangular area corresponds to the duplicated region of the four constant genes as reported by Flanagan and Rabbitts (1982). (**B**) Restriction map of the λpl8 *Xba*I clone 18.5 kb long (bold line) and 3′ region. Restriction enzymes: B = *Bam*HI, E = *Eco*RI, H = *Hin*dIII, X = *Xba*I. H* is the polymorphic *Hin*dIII site, generating the two fragments of the short allele: 10.5 kb (closed to Cα1) and 13.1 kb (Cα1 3′ flanking fragment). The probes used (2.1BB, 0.8SA, 2.3BB and 0.5BX) are shown in bold and underlined. (**C**) Schematic representation of the 5682 nucleotide sequence (the 3′ end of λpl8 *Xba*I clone) with Accession No. X76785 in the EMBL-GeneBank. The dotted box indicates the location of three dbEST clones of cDNA library of human B tonsil lymphocytes (Accession Nos AA215369, AA281085, AA215505). Vertical lines indicate the 20-bp tandem repeated sequence. Oblique lines indicate the three open reading frames localization. Vertical bold lines indicate the GA repeat region lying within the grey box indicating the purine-rich area. Map distances are expressed in kilobases.

transcripts (ibid.). These results suggest that the region is not only a *cis*-acting LCR, but probably contains a tissue-specific transcribed gene. Subsequently, it was possible to map three dbEST clones from human tonsil and mouse lymph node cDNA libraries within the 5682-bp sequence at the 3′ end of λpl8 clone (Fig. 1C), bolstering the hypothesis of the presence of a gene. In this paper, we demonstrate the association of this polyadenylated transcript to polysomes which indeed can be translated.

2. Materials and methods

2.1. Sub-cloning of λpl8, DNA sequencing, probes and PCR primers

The λpl8 *Xba*I clone (Gualandi et al., 1995) was subcloned in *Bam*HI, *Pst*I and *Apa*I subclones in pBluescript KS$^+$ (see Fig. 1B) and analysed with a Perkin-Elmer (Palo Alto, CA) capillary sequence automatic method. The subcloning was controlled for correspondence to the DNA of λpl8 and genomic length. The probes used were 2.1BB, 0.8SA, 2.3BB and 0.5BX subclones of λpl8 shown in Fig. 1B; probes of chicken β-actin and human ferritin genes were chosen for Northern control experiments. The PCR primers to amplify the *Hin*dIII polymorphic region were designed from the following sequence (GenBank Acccession No. Y14406): 5′: TCGGTTGAGGGCACCATTACAT; 3′: AAAGCGTGGGTGCCAGGTAT.

2.2. Southern analysis

Genomic DNAs were extracted by a standard method (Maniatis et al., 1989), digested with the restriction enzymes purchased from Boehringer (Germany), electrophoresed with TAE buffer in 0.4% agarose gel and capillary-blotted on Nylon Nucleobond together with 0.5 M NaOH. Hybridization was performed using probes labelled with a random priming technique using the non-radioactive luminescence method with the material and protocol of the Boehringer Dig. System.

2.3. Northern and polysome analysis

The procedures for cell lysis, sucrose gradient sedimentation of polysomes and analysis of the polysome/mRNP distribution of mRNA are discussed in Meyuas et al. (1996). The cells of the CLF-1 lymphoblastoid line, grown in a Petri dish (Ø = 200 mm from Falcon company) with 10 ml of RPMI 1640, with fetal calf serum 10%, glutamine and gentamycin in logarithmic phase, were harvested at a concentration of

0.5×10^6/ml. The cells were lysed, treated and the lysates centrifuged in sucrose gradient according to the low absorbance background procedure (Camacho-Vanegas et al., 1995). Ten fractions were collected from the centrifuged gradient, while monitoring and plotting the optical density on a diagram at 254 nm, and then ethanol-precipitated overnight at −20°C. The total RNA was extracted from cells or gradient fraction pellets using the RNAzol produced by Sigma (St. Louis, MO). For Northern analysis, RNA was fractionated on formaldehyde-agarose gel and transferred on a Gene Screen plus membrane (NEN–Dupont, Wilmington, DE). The filters were probed with the 0.8SA (Fig. 1B), chicken β-actin and human ferritin probes. Radioactive probes were prepared using the random priming technique (Maniatis et al., 1989) and hybridization performed as suggested by the NEN Gene Screen plus protocol.

2.4. Image analysis

The autoradiographic images were acquired using 2 scan Agfa scanner Adobe Photoshop (TIFF) and analysed using gel Pro analyser 2.0 of Medi Cybernetic LP. The molecular weight of the restriction fragments were calculated by automatic program interpolation with the appropriate molecular weight standards.

3. Results

3.1. Conservation of Cα1 and Cα2 3′ regions and their organization

To analyse the structure and possible rearrangements within the *Xba*I λpl8 clone (Fig. 1B; Gualandi et al., 1995), we sequenced 5682 bp (EMBL X76785) at the 3′ end of Cα1 and checked the correspondence of the subclones with the germline genomic DNA (Fig. 1B). The structure of the 5682-bp fragment is shown schematically in Fig. 1C, where more than 80 tandem repeats of a 20-bp consensus (Gualandi et al., 1995), a polypurine region including the GA repeats, three ORFs and the location of three reported dbEST clones (see Section 3.2) are indicated.

To compare the structure of the λpl8 clone to germline DNAs, we carried out a Southern blot hybridization analysis, which revealed a polymorphic pattern of this region in the donor DNAs. In particular, the presence of a *Hin*dIII polymorphism in the Cα1 region (results shown in this section) turned out to be informative in that one short allele (10.5 kb, Fig. 2A, lane 2), resulting from the presence of an additional *Hin*dIII site corresponded to the split of the Cα1 high-molecular-weight band characterizing the long allele (23.7 kb, Fig. 2A, lane 1). The 2.3BB or 0.5BX probes revealed that both fragments originated from the polymorphic *Hin*dIII site,

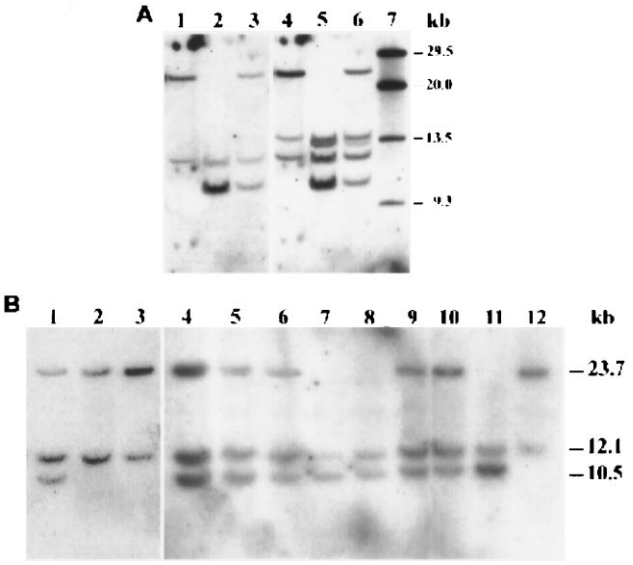

Fig. 2. Southern blot of human genomic DNA *Hin*dIII digestions. (**A**) Three DNAs, Mato, BNC and EL-7, were hybridized (lanes 1, 2, 3) with 2.1BB probe, and the same filter (lanes 4, 5, 6) rehybridized with probe 0.5BX. In lane 7, the λ EMBL12 was digested with *Bam*HI. With the 2.1BB probe hybridization, all DNAs display the 12.1-kb band corresponding to Cα2, and the 23.7 kb corresponds to the Cα1 long allele present in Mato (lane 1), where the BNC DNA has the 10.5-kb short allele fragment (lane 2). The El-7 DNA is heterozygous for the *Hin*dIII site in Cα1 since it has both fragments with 23.7- and 10.5-kb fragments (lane 3). The same filter hybridized with 0.5BX probe shows the Cα1 region with the polymorphic *Hin*dIII site. The 23.7-kb band present in lane 4 (Mato) appears now as the sum of the 10.5-kb plus the 13.1-kb new band of lane 5 (BNC). The common bands in lanes 4 and 5 are the Cα2 (12.1 kb) with the flanking of the duplicated region (13.7 kb). EL-7 DNA in lane 6 shows all the bands of lanes 4 and 5 displaying a heterozygous condition. (B) Hybridization with probe 2.1BB. Lanes: 1, CLF-1; 2, Daudi; 3, Raji; 4, Ramos; 5, BK013; 6, BK020; 7, BK021; 8, BK075; 9, BK078; 10, AND; 11, BNC; 12, PRF. In lanes 2, 3, and 12, three homozygous samples are shown with a long Cα1 allele (23.7 kb), in lanes 7, 8, and 11, three samples with a short Cα1 allele (10.5 kb) are shown, and in lanes 1, 4, 5, 6, 9, 10, the six samples are heterozygous.

thus indicating the presence of a duplicated flanking region hybridizing to both probes (there were no *Hin*dIII restriction sites within either of the probe fragments). The duplicated region 3′ of Cα1 and Cα2 spans for more than 10 kb as revealed by Southern blot analysis of *Hin*dIII-digested genomic DNA with the 2.3BB probe (Fig. 2A and B). All sequences in triplicate display a high level of conservation: in the autoradiographies, there are no differences in the intensities of the signals, even under a very high stringency of hybridization (Fig. 2A, lanes 1, 2, 3). The Southern blot analysis with different restriction enzymes allowed a map of the Cα1 3′ region to be constructed, as depicted in Fig. 1B. The pattern of 12 DNAs both from donors and lymphoblastoid cell lines digested with *Hin*dIII and hybridized with 2.1BB probe demonstrates that there is a widespread incidence of both alleles (Fig. 2B). This polymorphism

turned out to be a transition from guanine to adenine in the *Hin*dIII site lost in the long allele.

3.2. Analysis of dbEST clones

Fasta and Blast algorithms were used to determine whether the 20-bp repeat sequence present in the poly(A)$^+$ transcript could be found in the dbEST databank at NCBI center. Three dbEST clones from a human cDNA tonsillar library (Accession Nos. AA215369, AA281085 and AA215505) were found to have more than 95% homology with clone *Xba*I λpl8 (and therefore with the sequence flanking Cα1) and less than 80% with that flanking Cα2. The transcription direction of the three dbEST clones is the same as that of the immunoglobulin genes within the same cluster (Fig. 1C). Unfortunately, the largest of the three clones is only 650 bp long and contains a contiguous sequence of only one exon. Consequently, we were unable to identify the next exon or to find the 5′ of the gene. In the 358-bp-long mouse lymph node dbEST clone (Accession No. AA260818), there is a stretch of 80 bp, 73% homologous to the three human dbEST clones. The remaining 278 bp are probably part of a different exon.

3.3. Expression of the 20-bp repeats

The identification of these cDNA dbEST clones strongly suggests that this region is not only transcribed, as for any other heavy chain gene sterile germ-line transcripts (Lebman et al., 1990; Mills et al., 1990), but that the transcript is also polyadenylated. We have previously reported the presence, in CLF-1 lymphoblastoid cells, of appreciable amounts of a 4.7-kb poly(A)$^+$ RNA containing the eighty 20-bp repeats that are not related to any other IG transcripts, whether mature or not (Gualandi et al., 1995). To determine whether this RNA is also translated, we have analysed its polysome association (Setzer et al., 1980; Brannan et al., 1990). For this purpose, a cytoplasmic extract was prepared from growing cells of the lymphoblastoid CLF-1 line and fractionated by sucrose gradient sedimentation. The top panel of Fig. 3A shows the absorbance (254 nm) polysomal profile, and the bottom panel shows the Northern blot analysis of the RNA extracted from the 10 fractions collected from the sucrose gradient. Hybridization with the 0.8SA probe clearly shows that the 4.7-kb RNA forms a sediment together with polysomes. The same RNAs were controlled for both β-actin and ferritin genes in terms of their association with polysomes, confirming for the former the presence on the complexed form and, for the latter, only the presence in the unassembled fractions of ribosomes. To confirm that the high sedimentation rate of the 4.7-kb RNA is indeed due to the association with polysomes, we performed the usual appropriate control by treating the

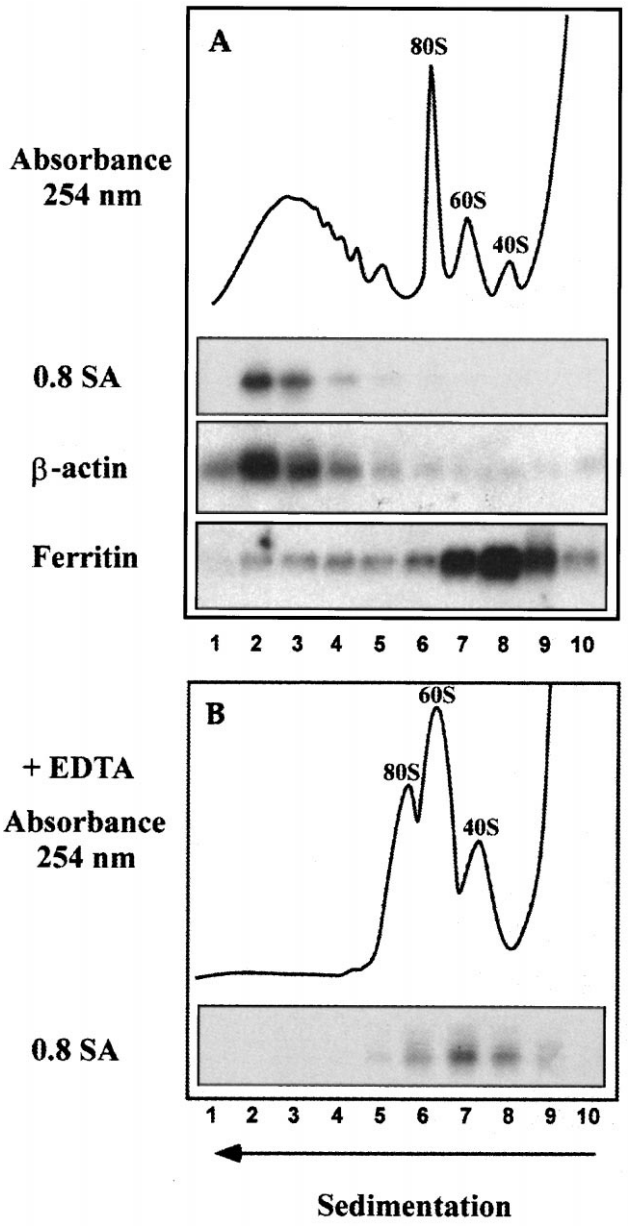

Fig. 3. Association with polysomes of the 4.7-kb mRNA. Cytoplasmic extracts, prepared from exponentially growing cells of CLF-1 lymphoblastoid line, were (**A**) not treated or (**B**) treated with EDTA and then fractionated by sucrose gradient centrifugation in 10 fractions: from 10 to 1, the direction of sedimentation of the gradient, where fraction 1 is the heaviest with the assembled polysomes. The top panels show the polysome profiles (absorbance at 254 nm) recorded during gradient fraction collection. RNA was then extracted from the gradient fractions and analysed by Northern blot hybridization (bottom panels) with the 0.8SA probe containing the 20-bp repeats. The RNAs extracted from the 10 gradient fractions, in the absence of EDTA, were also hybridized with a probe for chicken β-actin gene associated with polysomes and a probe for human Ferritin gene not associated with polysomes in the CLF-1 cell line.

extract with EDTA, before sucrose gradient centrifugation, under conditions that are known to cause polysome disassembly (Baierlein and Infante, 1974; Cardinali et al., 1987). The top panel of Fig. 3B shows that in

fact the EDTA treatment resulted in a complete dissociation of polysomes in ribosomal monomers and subunits; the bottom panel shows a displacement of the 4.7-kb transcript to an upper zone of the gradient, which is expected to occur with mRNAs upon dissociation from polysomes. The high sequence homology between the DNA fragments containing the 20-bp repeats flanking Cα1 and Cα2, does not allow us to determine, by hybridization with the probe 0.8SA, whether the transcript associated with polysomes is transcribed from the sequence at the 3′ end of Cα1 gene or from that at the 3′ end of Cα2.

4. Discussion

As more knowledge has been acquired about the organization of the regulatory region mapping at the 3′ end of both Cα1 and Cα2, a greater number of different roles and possible complex functions have been assigned to this region. Some of the information reported in this paper goes in this direction. In this respect, the polymorphism described here and in Pinaud et al. (1997) will be informative in terms of both function and possible differences, within the population, in the immunological response so as to determine how the gene/s in individuals who are polymorphic in this region is/are expressed.

Sequence analysis of this region indicates a variegated structure. In fact, on the strand transcribed, there is a purine-rich (polypurine) region (80% in a 360-bp stretch, starting around 3440 bp of our sequence) and located in a G+C-rich area (Fig. 1C). A purine-rich sequence is also present in the third intron of the human IG Cα1 containing a repeated motif with a pyrimidine at every sixth purine (Liming et al., 1990). In the 5682-bp sequence of the *Xba*I λpl8 clone, this structure lies just 5′ to the G+C-rich area and contains GA repeats (Fig. 1C) that play a key role in the activation of heat-shock-responsive elements (Glaser et al., 1990; Qin et al., 1993). In addition, the polypurine stretch suggests the possibility of DNA triple helices (triplex) formation (Usdin and Furano, 1989; Grabczyk and Fishman, 1995) that might be important both in regulating the trascription, by acting upstream of the transciption units (Grabczyk and Fishman, 1995), and in DNA recombination, by acting with other non-B DNA structures to promote illegitimate recombination (Hoffman-Liebermann et al., 1986; Usdin and Furano, 1989). In this respect, it seems noteworthy that such a potential triplex structure is about 1.8 kb apart from the EBV integration site in RGN1 cell line (Gualandi et al., 1992). The presence of a polypurine/polypirimidine sequence having a mirror symmetry (inverted repeats) gives further support to the hypothesis that the region is able to adopt a non-B DNA structure.

We have previously suggested that this region located at the 3′ end of Cα1 contains, besides the *cis*-acting structures evidenced by several groups (Chen and Birshtein, 1997; Mills et al., 1997), a new unknown gene unrelated to immunoglobulins (Gualandi et al., 1995). In the present work, we have shown that the independent poly(A)$^+$ RNA transcribed from this region is associated with polysomes, which is a strong evidence for active translation. This is the first gene found to lie within the immunoglobulin constant genes and having a different role with respect to the surrounding genes. In fact, the 4.7-kb mRNA associated with polysomes does not share any sequence with immunoglobulin mRNAs, as shown by the fact that no common transcript is evidenced by hybridization with the 0.8SA, the V(D)J region and the Cα1 exon probes. Moreover, the transcript size of 4.7 kb does not correspond to that of the germline or rearranged transcripts of other IGH isotypes (Lebman et al., 1990; Mills et al., 1990). These control experiments were necessary since in the 4.7-kb mRNA and in the dbEST clones, the transcribed strand is the same as that of the immunoglobulins. From the high sequence homology between the 3′ regions of Cα1 and Cα2 genes, it is not possible to determine from which of the two the mRNA is transcribed, although the three human dbEST clones have the highest homology with the Cα1 3′ sequence.

Besides the rearrangements of the V(D)J sequences and class switching, the B cells have to perform several functions to adequately produce the immunoglobulins during the different maturation steps (Serwe and Sablitzky, 1993; Cogne' et al., 1994). As already reported (Gualandi et al., 1995), in two of the four B cell lines examined, where the Cα switch took place, we found a much greater amount of mRNA containing the 0.8SA probe with respect to the other two. Although this observation needs to be supported by more data, it suggests the possibility that this gene is upregulated with immunoglobulin A (IGA) production. Furthermore, the fact that, in this same region, three dbEST clones isolated from a human B lymphocyte tonsillar cDNA library and one from mouse lymph node have been mapped strengthens the hypothesis that this gene is tissue-specific, being expressed in differentiated cells producing immunoglobulins.

It is noteworthy that a potential heat-shock-response element has also been identified at the 3′ end of the 5682-bp sequence of the λpl8 clone (Mills et al., 1997), suggesting the possibility that another regulatory path could be induced in B cells in stress-provoked conditions. It should also be noticed that the 495-bp-long open reading frame present in the 20-bp tandem repeat region codes for a polypeptide with a 34% homology to a repeated sequence at the carboxy-terminus of the major subunit of the RNA poly II enzyme described as having a regulatory function. This homology reflects a conservation of a repeated array of prolines and serines, probably responsible for a structure implicated in the interaction with DNA.

Up to now, attempts to isolate a full-length cDNA have not been successful. Considering the difficulties encountered in cloning the genomic sequence containing the 20-bp tandem repeats of Cα1 and Cα2 (Chen and Birshtein, 1995; Kang and Cox, 1996), it is not surprising that this cDNA is also unstable in a plasmid, thus requiring the adoption of a different approach that could probably involve RT-PCR. These results show that it might be worth characterizing this region of the genome further, searching for other genes involved in immunoglobulin production regulation.

Acknowledgements

We thank Dr. Stefania Bortoluzzi of the Biology Department of the University of Padova, for her fruitful help in identifying new dbEST clones at the Genome Data Bank. We are grateful to Prof. Enrico Calef who discussed with us the hypothesis and stimulated us to search for the presence of a gene in this regulatory region of the immunoglobulines. We thank Prof. Francesco Amaldi for his suggestions. We thank Dr. Maria Lo Ponte for revising the paper. We would also like to thank Dr. Palma Mattioli for elaborating the figures.

The research was financed by CNR contract n. 95.00631 GG and MURST 40% grants.

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