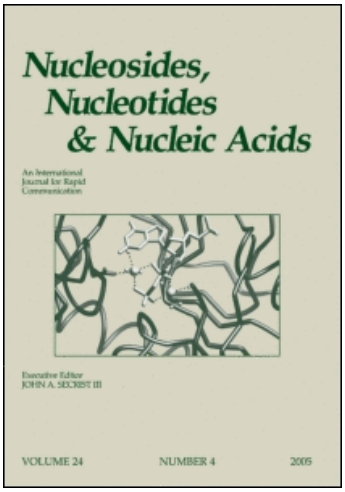

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Multiple Sequence Elements Involved in Polyadenylation at The Murine Igm Secretory Poly(A)Site.

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MULTIPLE SEQUENCE ELEMENTS INVOLVED IN POLYADENYLATION AT THE MURINE IgM SECRETORY POLY(A) SITE.

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ABSTRACT We show that the mouse immunoglobulin (IgM) secretory poly(A) site contains an extended hexanucleotide sequence element and two downstream elements spanning GU-rich regions which all contribute to polyadenylation efficiency. The multiple elements may provide a flexibility to accommodate changes during the regulation of polyadenylation.

The IgM heavy chain locus contains two poly(A) sites which are alternatively used (1-3) (see Figure 1A). The usage of the upstream secretory poly(A) site appears to be controlled by the efficiency of polyadenylation at this site making the secretory poly(A) site a good model to study the regulation of polyadenylation efficiency (4-6).

Polyadenylation sites are characterised by a highly conserved AAUAAA hexanucleotide sequence located approximately 20 nucleotides upstream of the site of cleavage and poly(A) addition (reviewed in (7)). In addition, unconserved GU-rich regions are often present downstream of the cleavage site and the sequence of the GU-rich regions as well as the spacing between them and the site of cleavage have been shown to play a vital role in determining the strength of the poly(A) site (reviewed in (8)).

The polyadenylation/cleavage complex which binds the hexanucleotide sequence and the downstream regions and is responsible for the cleavage and polyadenyltion, consists of a number of multi-molecular components including Cleavage/Polyadenylation Specificity Factor (CPSF), which binds the hexanucleotide sequence and Cleavage Stimulation Factor (CstF), which binds both CPSF and downstream GU-rich regions. (reviewed in (9,10)).

The consensus hexanucleotide sequence of the μ secretory poly(A) site is embedded in an AU-rich sequence and there are two GU-rich regions 8 nt and 11 nt in length, which are located 21 nt and 58 nt downstream of the hexanucleotide sequence, respectively (see figure 1B). We therefore examined the contribution of the extended hexanucleotide sequence and the two GU-rich regions to polyadenylation efficiency at the secretory poly(A) site *in vivo* in transfection experiments. We show that the multiple elements of the secretory poly(A) site all contribute to polyadenylation efficiency at this site.

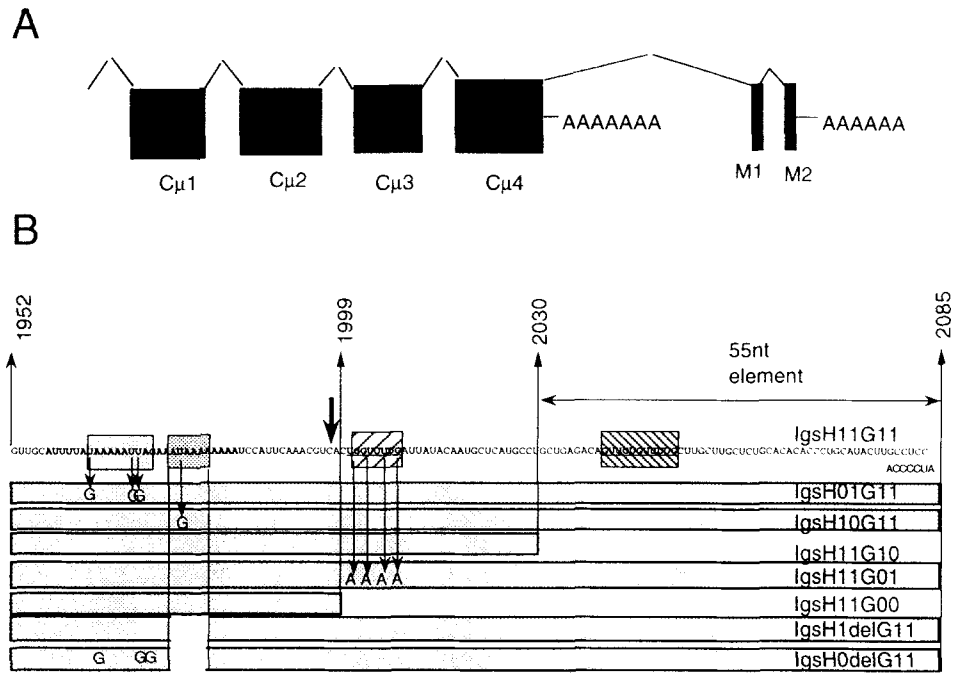

FIG 1 (A) The IgM heavy chain pre-mRNA can be alternatively processed into two forms of mRNA (B) Schematic Diagram showing the sequences surrounding the μ-secretorypoly(A) site and the mutant constructs used. The multiple hexanucleotide (shaded boxes) and multiple GU-rich regions (hatched boxes) are as follows: light shaded box: upstream adjacent AU-rich region, dark shaded box: consensus hexanucleotide sequence, light hatched box: proximal GU-rich region, dark hatched box: distal GU-rich region. The AU-rich region surrounding the two shaded boxes is marked in bold. The cleavage site is indicated with a bold arrow. The 55nt element is indicated between the arrows. Names of the mutant constructs indicating the mutations or deletions which they contain are indicated.

MATERIALS AND METHODS

Plasmids and RNA substrates: PCR products from the secretory polyA site and surrounding sequences, containing mutations, a 5′Bgl II site and a 3′Xba I site introduced as part of the synthetic primers, were cloned into pGem T vector. Inserts were subcloned into pPKLT55 which contains the luciferase reporter gene under control of the HSV-2 promoter (Dietrich-Goetz et al, manuscript in preparation) in place of the HSV-2 poly(A) site between the Bgl II and Xba I sites.

Cell culture and transfection J588L is a plasmacytoma which has lost endogenous expression of its immunoglobulin genes (11). This was obtained from the European

Collection of Animal Cell Cultures (Salisbury, U.K.) and maintained in Dubbecco's modified Eagles medium with 10% foetal calf serum, penicillin and streptomycin. Plasmids containing the luciferase gene followed by wild type or mutated sequences spanning the secretory poly(A) sites were transfected into J558L cells according to Grosschedl and Baltimore (12). After 48 hours cell extracts were made and luciferase activity was measured using a Luciferase Assay System (Promega cat. no. E4030) and a luminometer, MicroLumat LB 96P (Berthold). Transfection efficiency was measured by co-transfection of SVCATb. CAT activity was measured by incubation with ^{14}C Chloramphenicol and Buryryl CoA followed by extraction with tetramethylpentadecane: xylene (2:1) and measurement of ^{14}C in the organic phase in a β counter.

RESULTS

An extended hexanucleotide sequence and both GU-rich regions to contribute to μ-secretory poly(A) site efficiency *in vivo*. We examined the contribution of the hexanucleotide sequence and the two downstream GU-rich regions to polyadenylation efficiency at the secretory poly(A) site *in vivo* using the expression vectors containing the luciferase gene followed by the secretory poly(A) site containing deletions or mutation in each of the elements under investigation (see figure 1 B for details) and transfected them into J558L plasmacytomas (Figure 2).

When the central U is mutated to G in the consensus hexanucleotide sequence (see Figure 1B), a mutation which abolishes the activity of the hexanucleotide sequences in a number of other poly(A) sites (13), luciferase activity is reduced to 20% of that of the wild type sequence (Figure 2, compare lanes 1 and 2). This activity can be reduced significantly further by additionally mutating the 3 U's to G's in the AU-rich sequence upstream of the consensus sequence (Figure 2 compare lanes 2 and 3), suggesting that the AU-rich sequence can maintain a low level of polyadenylation activity in the absence of a functional consensus sequence. Similarly, even if the whole consensus hexanucleotide sequence is deleted, residual polyadenylation activity is retained (Figure 2 see lane 4) which can be again reduced by additionally mutating the 3 U's to G's of the adjacent upstream AU-rich region (Figure 2, compare lanes 4 and 5).

Deletion of a 55nt element containing the distal GU-rich region reduced polyadenylation activity by 50 to 80% (Figure 2 compare lanes 1 and 6). Similarly mutation of the proximal GU-rich element while retaining the distal GU-rich region (see Figure 1 B) reduced polyadenylation activity to a similar extent (figure 2, compare lanes 1 and 7). However, deletion of both GU-rich regions abolished polyadenylation activity completely (Figure 2, lane 8). Thus both GU-rich regions contribute to polyadenylation efficiency and both are necessary to restore full activity.

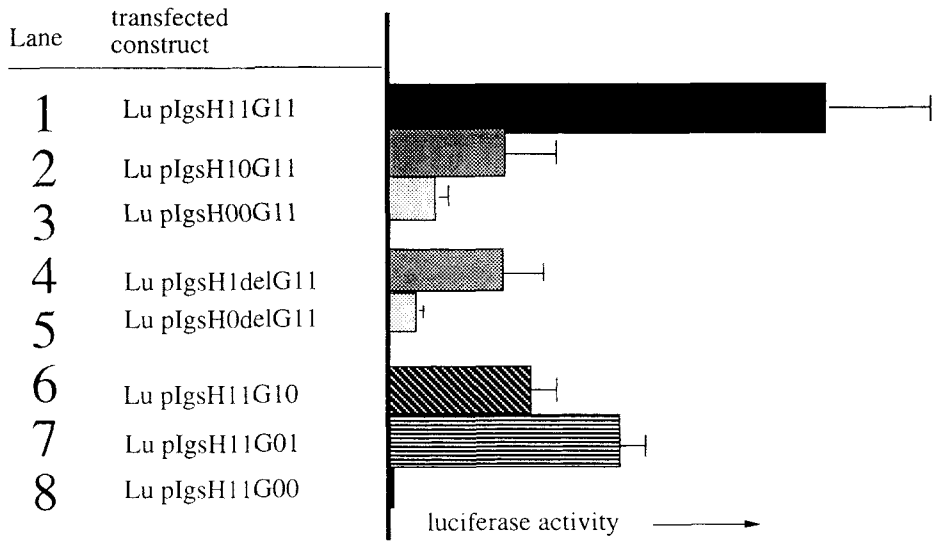

FIG 2. Contribution of the hexanucleotide sequence and the proximal and distal GU-rich regions to polyadenylation efficiency *in vivo*. The respective sequences were inserted downstream of the the luciferase gene at the poly(A) site of pPkLT55 and transfected into J558L plasmacytomas in triplicates. Transfection efficiency was standardised by co-transfection with SVCATb. Luciferase activity was measured for each construct and expressed as % activity of a standard +/- s.e. Bars represent % activity for (1) wild type; (2) consensus hexanucleotide mutation U-G, (3) mutation of 4 U's to G in AU-rich region (4) deleted consensus hexanucleotide (5) deleted consensus hexanucleotide and mutation of 3 upstream U's to G (6) Deletion of 55nt spanning the distal GU-rich region (7) Mutation of proximal GU-rich regions (8) Deletion of sequences containing both GU-rich elements

DISCUSSION

A number of worker have detected changes in the polyadenylation complex which correlate with differing levels of usage of the secretory poly(A) site. These include an inhibitory factor which specifically targets the μ-secretory poly(A) site and a factor which enhances polyadenylation activity at a number of poly(A) sites (14,15). We have previously found a 28-32 kDa polypeptide whose induction correlates with the increased usage of the secretory poly(A) site and whose binding is dependent upon the 55nt element spanning the distal GU-rich element (16). Indeed, it has been suggested that the regulation of polyadenylation efficiency may be as complex as that of transcription initiation in which common and specific factors may inhibit or enhance a particular polyadenylation reaction (9). We suggest that the flexibility in binding of the core components of the polyadenylation complex at the μ-secretory poly(A) site would allow it to bind these

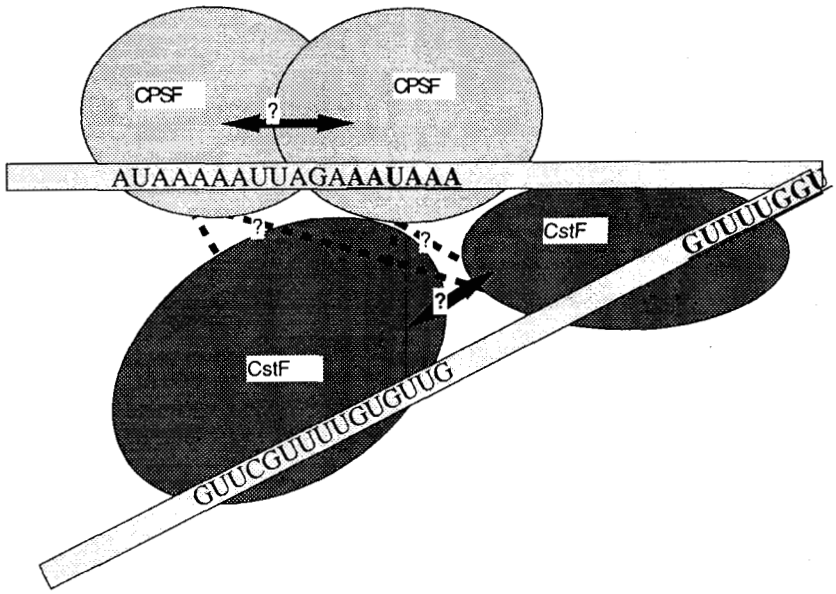

FIG 3 Schematic diagram of the model for interaction of CPSF and CstF with the repeated sequence elements of the μ-secretory poly(A) site.

multiple factors at different times during B cell development and provide multiple elements at which pre-initiation complex binding can be enhanced and inhibited by transacting factors (see Figure 3 for model).

The μ-secretory poly(A) site appears to be unique in having multiple elements as the gamma, epsilon and alpha loci contain a single hexanucleotide sequence and a single optimally placed downstream GU-rich element if it is present. These different structures raise the possibility that the alternative processing of IgM heavy chain pre-mRNA is regulated in a different way to that of isotypes in which the production of high affinity secreted antibody is the major consideration.

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