Genomic Cloning and Characterization of the Human Eukaryotic Initiation Factor-2β Promoter*

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The translation initiation factor eIF2 consists of three subunits that are present in equal molar amounts. The genomic DNA containing the gene for eIF2β and its promoter were cloned and sequenced to characterize further the mechanism of their regulated synthesis. Whereas Southern blot analysis indicated that a number of copies of the gene may exist, only one full-length intron-containing copy was identified. Similar to the eIF2α promoter, the eIF2β promoter is TATA-less, CAAT-less, and GC-rich and contains an α-Pal binding motif. Mutation of the α-Pal binding sequence resulted in an 8-fold decrease in activity when assayed by the luciferase reporter gene constructs. The data suggest a common mechanism of transcriptional control for the two cloned subunits of eIF2.

The translation initiation factor eIF2 catalyzes the first regulated step of protein biosynthesis, the binding of the initiator Met-tRNAi to the 40 S ribosomal subunit. Binding occurs as a ternary complex of Met-tRNAi-eIF2-GTP. All three subunits of eIF2 (α, β, γ) are required for the catalytic utilization of eIF2 during protein synthesis initiation (1, 2). The α subunit of eIF2 is a 36.2-kDa polypeptide whose phosphorylation state regulates activity of the heterotrimer (3). The β subunit appears to bind GTP or GDP and is a 51.9-kDa polypeptide (4). The 38.3-kDa γ subunit may be directly involved in binding of the ternary complex to mRNA (5, 6). None of the three subunits appears to exist as a monomer outside the eIF2 heterotrimer. Initial experiments demonstrated that balanced synthesis of the α and β subunits is predominantly the result of different rates of ribosomal elongation (7). Subsequent experiments suggested that eIF2α expression could be regulated by antisense transcripts that form double-stranded RNA during T-cell activation (8). Regulation of sense transcription is under the control of a transcription factor designated α-Pal, which binds to an unusual direct repeat element. α-Pal is homologous to the developmental transcription factors F3A2 and evg (9). Potential target genes of α-Pal can be broadly classified as encoding growth-responsive factors (9). To see if the eIF2β subunit was regulated via similar mechanisms, the eIF2β gene and its promoter region were cloned and characterized.

MATERIALS AND METHODS

Library Cloning Screening—The cDNA for eIF2β was a gift from J. W. B. Hershey (GenBank number M29536). The nucleotide sequence of the promoter region has been reported to GenBank (number AF076927). A human lung fibroblast genomic library was constructed in the λ phage vector. Lambda Fix was purchased from Stratagene (La Jolla, CA). Subgenomic libraries were constructed by digesting high molecular weight DNA from K562 cells (ATCC, Manassas, VA) to completion with the indicated restriction enzymes followed by separation on a 0.5% Sea Kem GTG-agarose gel (FMC, Rockland, ME) and staining with ethidium bromide, and was the region containing the fragment of interest excised. The DNA was then purified and ligated into prepared phage arms following the manufacturer’s instructions (Stratagene). To ensure the proper region of the gel was excised, the gel (minus the excised band) was Southern blotted and probed with the fragment of interest. Screening of these libraries was carried out essentially as described previously (10). Northern blots were prepared following standard procedures (10). Southern blots of genomic DNA were prepared following the instructions for use of the Zeta probe blotting membranes (Bio-Rad). All hybridizations were done in Church-Gilbert buffer (0.5 M NaPO4, pH 7.0), 1% bovine serum albumin, 7% sodium dodecyl sulfate, 10 mM EDTA) for 18 h at the indicated hybridization temperature and washed with a final stringency of 0.15 M NaCl for 1 h at the indicated hybridization temperature.

Ribonuclease Protection Assays—Ribonuclease protection assays (RPAs) were performed as described by the manufacturer (Ambion, Austin, TX). Riboprobe were generated using the Maxiscript in vitro transcription kit according to the manufacturer’s instruction (Ambion). The size of the protected fragments was determined by comparison with control RNA and by comparison with a deoxy sequencing ladder.

Luciferase Assay—Luciferase assays were performed as described previously (11). Briefly, NIH 3T3 cells were plated in 6-well plates at a density of 1 × 10⁶ cells/well and transfected using LipofectAMINE (Life Technologies, Inc.). After 48 h, cell lysates were prepared, and 5-µl aliquots were added to 100 µl of luciferase reagent and assayed in a Monolight™ 2010 luminescence meter for 10 s. Final results were given in units of luciferase activity/µg of protein.

RESULTS

A cDNA clone for eIF2β was isolated from human liver mRNA, and the encoded polypeptide was shown to interact with the eIF2α and γ subunits (6). Southern blots of DNA isolated from K562 cells (ATCC) probed with the eIF2β cDNA produced a large number of fragments, indicating the gene for eIF2β is either very large or there are multiple copies (Fig. 1A). Hybridization of duplicate Southern blots with either an oligonucleotide probe from the 3′-UTR (bases 1273–1298) or a random-primed fragment from the 5′-UTR (1–103) generated a simplified pattern of only 4 or 5 bands (Fig. 1, B and C, respectively). These results suggest the existence of multiple genomic fragments with a high degree of homology to the eIF2β cDNA. Because of the high degree of specificity of oligonucleotide probes compared with random-primed fragments, the 3′-UTR oligonucleotide probe was initially used to screen a human fibroblast genomic library.

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† The abbreviations used are: RPA, ribonuclease protection assay; UTR, untranslated region.
Genomic clones corresponding to three of the four bands detected using the 3'-UTR oligonucleotide probe were isolated after screening multiple libraries (Fig. 2). Although all three exhibited a high degree of homology to the cDNA, only one was nearly identical and contained introns. This clone is referred to as clone 1A (Fig. 2). Additional sequencing and restriction mapping showed that clone 1A contained an insert 13 kilobases in length and 550 bases of the cDNA divided into 5 exons. A full-length clone was obtained by using polymerase chain reaction to amplify the DNA between the last exon in clone 1A and upstream exons. The promoter region and initiation codon were cloned by creating subgenomic libraries of the five DNA fragments that hybridized to the 5'-UTR (1–103) of the cDNA and probing with a random-primed probe from the 5'-UTR (1–103).

**Fig. 1.** eIF2β Southern analysis. A, hybridization of K562 genomic DNA with random-primed cDNA. M, markers; 1, 10 µg of DNA digested with EcoRI; 2, 10 µg digested with HindIII. B, hybridization of K562 genomic DNA with an oligonucleotide probe from the 3'-UTR (1273–1298). 10 µg of DNA was digested with the following enzymes: 1, EcoRI; 2, HindIII; 3, PstI; 4, XhoI; 5, EcoRI. C, hybridization of K562 genomic DNA digested with EcoRI and probed with a random-primed probe from the 5'-UTR (1–103).

**Fig. 2.** cDNA maps. Sequenced regions of the eIF2β genes are depicted below the published cDNA. The arrows indicate the beginning and the end of the coding region of the cDNA. The dots above the lines indicate the regions of sequence divergence from the published cDNA sequence.

Genomic clones corresponding to three of the four bands detected using the 3'-UTR oligonucleotide probe were isolated after screening multiple libraries (Fig. 2). Although all three exhibited a high degree of homology to the cDNA, only one was nearly identical and contained introns. This clone is referred to as clone 1A (Fig. 2). Additional sequencing and restriction mapping showed that clone 1A contained an insert 13 kilobases in length and 550 bases of the cDNA divided into 5 exons. A full-length clone was obtained by using polymerase chain reaction to amplify the DNA between the last exon in clone 1A and upstream exons. The promoter region and initiation codon were cloned by creating subgenomic libraries of the five DNA fragments that hybridized to the 5'–103 base pairs of the cDNA as shown in Fig. 1C. From the subgenomic library constructed from the 2.3-kilobase fragments, one clone was isolated that contained the missing cDNA sequence and could be linked to clone 1A. This produced a full-length cDNA and is referred to as clone 1 (Fig. 2). In addition, another pseudogene was isolated from this library, which contained the whole open reading frame.

**Fig. 3.** Oligonucleotide hybridization conditions. Complementary oligonucleotide probes corresponding to the divergent region in the coding region of the cDNA (991–1008) and the corresponding sequence in clone 1 and clone 2 were synthesized and used to probe three panels of DNA dot blots. Each panel contained a dot of 100 ng or 10 ng of DNA of the cDNA clone 1 or clone 2 as indicated to the left of the figure. The oligonucleotide probe used to probe the panel is indicated at the top of the figure. Under stringent washing conditions, the oligonucleotide probes would only hybridize to their corresponding target DNA.

**Fig. 4.** Northern blot analysis. Similar hybridization and washing conditions to those used in Fig. 3 were used to hybridize and wash three Northern blots. The oligonucleotide probe used as a probe on the blot is indicated above the blot. A, cDNA oligonucleotide probe; B, clone 1 oligonucleotide probe; C, clone 2 oligonucleotide probe. Within each blot lane 1 contains 10 µg of K562 total RNA; lane 2, 0.250 µg of poly(A) RNAs K562 RNA; lane 3, 10 µg of mouse total RNA (CLONTECH); lane 4, 0.250 µg of mouse poly(A) RNAs (CLONTECH). A eIF2β message was only detected with the probe derived from clone 1. No signal above background was detected with the other two probes. D, as a size control an additional Northern blot was probed with a random-primed probe of the entire cDNA. Lanes 1–3 contain 5, 10, and 20 µg of K562 total RNA, respectively.
frame with a number of point mutations but lacked introns. This clone is referred to as clone 4 (Fig. 2).

The sequence of clone 1 differed from the published cDNA in two respects. The 5′ most 18 bases reported to be in the cDNA were not present in the 2.3-kilobase promoter fragment. Clone 1 also differed from the cDNA at position 1000. The cDNA contained a T at this position and the genomic clone contained a C. This difference could be the result of a mutation introduced by reverse transcriptase during the cloning of the eIF2β cDNA or it could indicate that more than one copy of the sequence. The length of this band is correspondingly...

hybridize to the Northern blot (Fig. 4A), the T at position 1000 may be an artifact introduced during the cloning process of the cDNA.

To define the sequence at the 5′ end of the message, total K562 RNA was hybridized with radiolabeled antisense riboprobes derived from either the 5′ end of the cDNA or clone 1 and analyzed in a RPA. As a size control, the antisense riboprobes were also hybridized with a non-radiolabeled sense fragment transcribed from the original eIF2β cDNA. Fig. 5A (lane 3) shows that incubation of the radiolabeled cDNA probe with the cDNA sense control gave a band that was the correct length for a fully protected cDNA fragment. As a point of reference the length of this band is defined as 1 (Fig. 5, A and B). Incubation of the cDNA sense control message with the clone 1 riboprobe produced a band 18 bases shorter that corresponds to the point of divergence for the cDNA sequence and the genomic sequence. The length of this band is correspondingly −18 (Fig. 5, A, lane 11, and B). Incubation of the cDNA probe with total K562 RNA also produced bands of approximately 18 bases shorter than the cDNA/cDNA hybrid, indicating that the 5′-terminal 18 bases contained in the cDNA are not present in the pool of K562 RNA (Fig. 5A, lanes 4–6). Hybridization of the clone 1 riboprobe with total K562 RNA generated bands approximately 21 bases longer than the cDNA, indicating that the genomic sequence of clone 1 was again correct and that the 5′-UTR was longer than previously reported (Fig. 5A, lanes 13–15). Because the cDNA was isolated from a library derived from liver mRNA (6), this difference could be tissue-specific. However, hybridization with liver RNA with each probe gener-
Genomic Cloning of the eIF2β Promoter

Fig. 6. Primer extension analysis of eIF2β mRNA in K562 cells. Total RNA from K562 cells was isolated and annealed with a 5' end-labeled non-coding strand primer that hybridizes to bases 70–87 of the cDNA. This primer was extended with reverse transcriptase (M-MLV); the resulting product was isolated and resolved on a denaturing polyacrylamide gel as described under "Materials and Methods." The size of the fragment was determined by comparing the resulting bands to a sequencing ladder (lanes labeled G, A, T, C). The extension reaction was carried out in the absence (lane 1) or the presence (lane 2) of actinomycin D (50 μg/ml).

Fig. 7. Genomic map. A, the exon portions of the eIF2β gene are depicted as boxes. Restriction endonuclease recognition sites are indicated. R, EcoRI; H, HindIII; X, XbaI; P, PstI. The numbers below correspond to the exon number. B, exon structure and minigene divergences. Column 2 of the table lists the nucleotides of the cDNA contained in each exon (column 1). The numbering is based on the published cDNA (6). Column 3 lists the amino acid changes in the minigene (clone 4) compared with the corrected cDNA (clone 1).

translation of clone 4 generated a protein product very similar in size to that translated from the cDNA (data not shown). Several attempts were made to identify a transcript using Northern blots, RPA of K562, and activated lymphocyte RNA, but none could be detected (data not shown).

The promoter region of eIF2β (−1000 to +1321) was searched for transcriptional elements and was found to contain a number of potential transcription factor binding sites (Fig. 8). The eIF2β promoter contains a consensus sequence for the α-Pal transcription factor at −25. This element is also present in the promoter of the α subunit of eIF2 (9). Positioned over the cap site is a potential Sp1 site with potential E2F, C/EBP, c-myc, and two SIF sites located downstream at +15, +22, +253, +93, and +106, respectively. Upstream of the cap site, potential p53, Ap2, myoD, F-Act1, and CAAT box motifs exist.

Previous research has demonstrated the importance of the α-Pal site in the regulation of the eIF2β promoter (1). To assess the importance of the α-Pal element in the expression of the eIF2β gene, luciferase reporter constructs were made that contained mutations in the α-Pal region of the promoter. The α-Pal sequence was mutated from TGCCGAGCCGCA to TGCAAATTGCAT, which had previously been shown not to bind the α-Pal transcription factor (7). This mutation decreased promoter function in the reporter constructs 8-fold compared with the wild type promoter (Fig. 9, A and B, column 2 versus 3). A similar change in activity was detected in transfection of both 3T3 and 293 cells. To detect possible antisense activity, the promoter region was cloned into the luciferase plasmid in both 3T3 and 293 cells. To detect possible antisense activity, the promoter region was cloned into the luciferase plasmid in the antisense orientation and transfection into 293 cells or 3T3 cells. No luciferase activity was detected using a region corresponding to the first intron (+125 to +1300) alone in the sense or antisense orientation (Fig. 9, columns 4 and 5, respectively). Furthermore, no luciferase activity was detected using the entire promoter region (−1000 to +1300) in the antisense orientation (Fig. 9, column 6). Experiments using reverse transcriptase-polymerase chain reaction of RNA isolated from G0 and activated T-cells also failed to detect any antisense RNA...
transcription within the first intron region of eIF2, which has been described for eIF2 (8) (data not shown).

**DISCUSSION**

Cell growth and differentiation require the regulated expression of a large number of proteins. One mechanism for achieving coordinate regulation is through the use of a common regulatory transcription factor. Examples of this theme are the CREB family of responsive genes and the NF-κB family. The presence of a functional α-Pal site in the two subunits of eIF2 that have been cloned indicates that α-Pal may also act as a coordinating factor.

Obtaining the genomic clone for eIF2β was much more complicated than for eIF2α. Multiple pseudogenes were identified for the eIF2β subunit as well as an intronless minigene. In contrast, eIF2α was encoded by a single gene (12). Furthermore, several differences were identified in the genomic clone compared with the cDNA. Through the use of high stringency Northern blots and RPAs, the genomic clone was shown to be the correct sequence, and no mRNA corresponding to the cDNA sequences could be detected. The differences in the 5′-UTR and those at base 1000 in the published cDNA compared with the genomic sequence are most likely the result of ligation artifacts produced during the creation of the libraries and poor fidelity of reverse transcriptase, respectively.

The minigene identified during the cloning may be the result of a retrotransposition event. Although in vitro translation of this open reading frame generated a protein that was very similar in size to that generated from the cDNA, no transcript for this gene could be detected in vivo by Northern blot analysis, RPA in K562, or T-cells. Other bands are present on Northern blots probed with the eIF2β cDNA, and these mRNAs are most likely the result of multiple polyadenylation sites as has been reported for other translation factors (12). Although it would appear that only one functional gene for eIF2β exists, multiple functional copies of other translation factors have been identified (13). Recently two genomic clones were isolated for eIF4E; one contained introns and the other was intronless (14).

Whereas eIF2α and eIF2β are regulated at the transcriptional level by α-Pal, the cis elements in the two promoters are distinct. The eIF2α subunit promoter has two adjacent sites, which bind α-Pal with different affinities. Mutation of the high

**FIG. 8. Promoter sequence.** The sequence of the eIF2β promoter region (−1000 to +200) is presented. An arrow indicates the start site of transcription as mapped by both primer extension and ribonuclease protection experiments, and the initiation codon is italic and boxed. The α-Pal transcription factor binding site is underlined. Other potential transcription factor binding sites are identified by boxes. The 5′-UTR sequence identified in clone 1, which was missing from the published cDNA, is indicated in **bold**.
affinity site decreases expression 12-fold, whereas mutation of the lower affinity site inhibits expression 3-fold. In contrast, eIF2β has only a single site whose deletion results in an 8-fold reduction in transcriptional activity. However, both genes are TATA-less and have the α-Pal sites positioned in the traditional −30 TATA box site. Although potential α-Pal binding sites have been identified in a number of other genes (9), their importance has yet to be demonstrated. Characterization of α-Pal binding sites identified in these other growth-responsive genes is essential to defining the role of α-Pal as a coordinating transcription factor involved in the regulation of expression of growth response genes.

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Genomic Cloning of the eIF2β Promoter

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