Role of Sequences within the First Intron in the Regulation of Expression of Eukaryotic Initiation Factor 2α*

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Resting human peripheral blood T cells synthesize proteins at very low rates and contain very low levels of eukaryotic initiation factor (eIF) 2α mRNA. During mitogenic activation, the level of eIF-2α mRNA increases at least 50-fold, an effect thought to be due primarily to intranuclear stabilization of the primary transcript (Cohen, R. B., Boal, T. R., and Safer, B. (1980) EMBO J. 9, 3831-3837). Analysis of sequences within the first intron revealed a region with homology to the "initiator" (Inr) sequence first described by Smale and Baltimore (Smale, S. T., and Baltimore, D. (1989) Cell 57, 103-113). This Inr element is positioned 450 bases downstream of the eIF-2α promoter and is oriented to generate an overlapping antisense transcript. Deletion or mutation of the Inr element decreases at a reproducible 5-8-fold increase in the activity of an eIF-2α promoter-driven CAT reporter gene and a corresponding 2.5-fold decrease in activity of an antisense driven luciferase reporter gene in vivo in 293 cells. In vitro transcription analysis also reveals antisense transcripts which depend on an intact Inr element and whose 5′ ends map to sequences surrounding the Inr consensus sequence. A potential role for double-stranded RNA generated by these overlapping divergent transcription units in the regulation of eIF-2α gene expression in T cells is suggested.

Resting (G0) T cells are metabolically quiescent and have undetectable levels of DNA synthesis and low levels of RNA and protein production (reviewed in Refs. 1 and 2). A rapid increase in protein synthesis is an early event during activation of T cells following antigenic stimulation or by mitogenic lectins such as phytohemagglutinin (2). Single ribosomes are recruited onto mRNA to form polyribosomes, and increased translational activity is seen within the first few hours of mitogenic activation (3, 4). The rate of protein synthesis increases during the first 24-72 h of activation and results in levels of protein synthesis that are at least 10-fold greater than the rate in G0 T lymphocytes (5, 6). This rapid increase in protein synthesis following mitogenic stimulation of quiescent human T cells occurs at the level of translation initiation (4, 7). Although eIF-2α mRNA is very well translated in both quiescent and activated T cells (8, 9), human G0 lymphocytes contain very low levels of eIF-2α message (10). During the first 24 h of activation, however, eIF-2α mRNA increases more than 50-fold. Neither changes in the rate of transcription of the eIF-2α gene nor changes in the half-life of the message appear to account for all of this rapid and large increase in eIF-2α mRNA. We have therefore hypothesized that stabilization of the nuclear precursor sufficient to allow processing and transport to the cytoplasm might account for the large increase seen with mitogenic stimulation (10).

Previous work in this laboratory has revealed a DNase I-hypersensitive site in chromatin mapping to the 5′ portion of the first intron of the gene encoding eIF-2α between +220 and +300 (11). Analysis of the sequence of this region revealed an element with perfect homology to the conserved sequence of the initiator (Inr) element first described by Smale and Baltimore (12, 13) as well as two TATA-like sequences. The Inr sequence (+448, see Fig. 3) and one of the TATA-like elements (TACAAT,+353) are oriented opposite the direction of transcription for eIF-2α. The second TATA-like element (TACAATAT,+361) is oriented in the same direction as eIF-2α. The presence of an Inr element oriented to generate a transcript opposite in direction from the eIF-2α transcript suggested that regulation of eIF-2α expression by an overlapping antisense transcript may account for the apparent change in stability of the sense primary transcript during mitogenic activation of T cells.

The specific aim of this work was to characterize in a preliminary fashion the effect of mutations to this region in the first intron of the eIF-2α gene. We find that elimination or mutation of the consensus Inr element results in a reproducible 5-8-fold increase in the activity of a reporter gene in vivo, both enzymatically and by Northern analysis, and a corresponding 2.5-fold decrease in activity of an antisense driven luciferase reporter gene. We also identify by in vitro transcription and primer extension analysis antisense transcripts whose start sites map to the Inr region in the eIF-2α gene and overlap the eIF-2α sense transcript for at least 450 bases.

**EXPERIMENTAL PROCEDURES**

**Construction of CAT Expression Vectors and Templates for in Vitro Transcription**—The plasmid vector pCAP2CAT and the sequence of the eIF-2α gene promoter have been described (11, 14). The BamHI-EcoRI (806 to +478) fragment of the eIF-2α promoter was cloned into pUC8 to create pUC8-CAT. It was inserted into the Smal site of the expression vector pCAP2CAT to create the expression vector pCAP2CAT-CAT.

**Transcription**—The transcription reactions were performed in a total volume of 20 μl containing 100 mM Tris-HCl pH 7.5, 75 mM KCl, 10 mM MgCl2, 2 mM dithiothreitol, 1 mM spermidine, 0.5 mM each of ATP, GTP, CTP, and UTP, 5 mM spermine, 0.1 μCi of [γ-32P]ATP (1000 Ci/mmol), and 10 ng of CAT expression vector DNA in the presence of 200 μg of HeLa S3 nuclear extract. After 30 min at 30°C, the reactions were stopped by heating at 65°C for 5 min. The products were analyzed by electrophoresis on a 6% polyacrylamide gel containing 7 m urea.

**RESULTS**

Mutations to the Inr element in the eIF-2α gene promoter have been shown to decrease transcription from this promoter (11). Deletion of the Inr element results in a reproducible 5-8-fold increase in activity of a CAT reporter gene (11, 14). Previous work in this laboratory has revealed a DNase I-hypersensitive site in chromatin mapping to the 5′ portion of the first intron of the eIF-2α gene (10). This Inr element is positioned 450 bases downstream of the eIF-2α promoter.

The Inr element is oriented to generate an overlapping antisense transcript which can be detected by Northern analysis and in vitro transcription. The antisense transcript is transcribed from both the eIF-2α gene and a corresponding 2.5-fold decrease in activity of an antisense driven luciferase reporter gene in vivo in 293 cells.

**DISCUSSION**

The results presented here suggest that regulation of eIF-2α expression by an overlapping antisense transcript may account for the apparent change in stability of the sense primary transcript during mitogenic activation of T cells. The specific aim of this work was to characterize in a preliminary fashion the effect of mutations to this region in the first intron of the eIF-2α gene. We find that elimination or mutation of the consensus Inr element results in a reproducible 5-8-fold increase in the activity of a reporter gene in vivo, both enzymatically and by Northern analysis, and a corresponding 2.5-fold decrease in activity of an antisense driven luciferase reporter gene. We also identify by in vitro transcription and primer extension analysis antisense transcripts whose start sites map to the Inr region in the eIF-2α gene and overlap the eIF-2α sense transcript for at least 450 bases.

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2. T. R. Boal, manuscript in preparation.

3. The abbreviations used are: eIF, eukaryotic initiation factor; Inr, initiator; CAT, chloramphenicol acetyltransferase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.

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Fig. 1. Relative CAT activities of eIF-2α-CAT hybrid constructs. Plasmids used for transient transfection assays are shown. * designates the transcription start site. A total of 10 μg of DNA (CAT constructs plus carrier PUC 8 and control vector pRSV-luciferase) was added for all constructs. 1.6 pmol (5-μg equivalent) of test DNA was used. 100 ng of the transcription control plasmid, pRSV-luciferase, was included in all transfections. DNA concentrations were measured by fluorometry (Hoefer/Hoechst) and concentrations confirmed by gel electrophoresis. The calcium-phosphate method was used for all transfections in which luciferase-containing plasmids were transfected. Cells were seeded at 5 × 10⁵/plate and transfected with a total of 15 μg of DNA including 10 μg of salmon sperm DNA as carrier and equimolar amounts of the test plasmids. Six hours after transfection, fresh medium was added, and the incubation continued for another 34 h. Cells were harvested at 48 h, and CAT and luciferase activities were analyzed as described previously (17–20).

Northern Blot Analysis—Total cellular RNA was prepared from transiently transfected 293 cells using a modification of the Chirgwin method (RNAzol, Cinna Biotech). A total of 10–15 μg of RNA from each sample was heated to 65 °C in 50% formamide, 20% formaldehyde in 0.02 M MOPS buffer and electrophoresed in a 0.8% agarose gel containing 0.02 M MOPS. RNA was capillary-blotted to Nytran membrane. Similarity of RNA amounts loaded and efficiency of transfer were assessed by ethidium bromide staining of the gel. A single-stranded DNA probe was generated by primer extension from an M13mp19 construct containing the first 250 bases of the CAT coding sequence. RNA blots were hybridized as described by Church and Gilbert (21, 22) for 36 h and washed as described.

In Vitro Transcription and Primer Extension Analysis—In vitro transcription analysis of the eIF-2α region was performed in a total of 25 μl containing 60 mM KCl, 20 mM (NH₄)₂SO₄, 6 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 5 mM creatine phosphate, 10% glycerol and 12 μl of K562 nuclear extract (50 μg). The DNA template concentration was 0.1 pmol/25 μl of reaction mixture, and ATP, GTP, and UTP were 100 μM. 5 μCi of [α-³²P]GTP (800 Ci/mmol) was added per reaction, and incubations were for 30 min at 28 °C. Concentrations of the transcription products was performed as described previously (23).

Primer extension analysis was performed as previously described (14).

RESULTS AND DISCUSSION

We evaluated the effect of the putative Inr element on the relative promoter strength of the eIF-2α gene by in vivo reporter gene assays and by in vitro transcription. We first demonstrated that the transcription initiation sites for the CAT constructs correlated well with the initiation sites mapped for the endogenous gene (data not shown). Initial studies using a construct truncated at +264 showed that
Fig. 2. 5' deletion analysis of the eIF-2α promoter region identifies an antisense RNA polymerase II transcript. A, A−E are 5' deletion mutations of the eIF-2α promoter generated by Bal31 digestion of the eIF-2α SstI-EcoRI genomic fragment (−1057 to +478). The most 3' of the eIF-2α CAP site cluster is designated "P". B, 32P-labeled run-off transcripts of templates A−E were analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography. Arrowheads identify the major in vitro transcription products (lanes A−E). Transcription of the adenovirus 2 major late promoter linearized by PstI, included as a positive control, generates a 536-base runoff transcript. A BstNI digest of SV40 was used to calibrate transcript size. By Northern analysis, a single-stranded antisense probe complementary to the first 250 bases of the CAT coding sequence (+478) hybridized to an appropriately sized 1.5-kilobase transcript. A BstNI digest of SV40 was used to calibrate transcript size. B, in vitro transcription analysis was performed as in B in the presence of α-amanitin, 5 ng/ml. bp, base pair(s).

Fig. 3. Mapping the 5' end of the in vitro antisense transcript. A, purified RNA from in vitro transcriptions performed as in Fig. 2 was hybridized to 0.25 pmol of the 5'-32P-labeled 18-nucleotide primer indicated in panel B. Primer extension products (lane 1) were analyzed on a 6% sequencing gel calibrated using a dideoxy sequencing ladder of human eIF-2α. The 1.5-kilobase SstI-EcoRI genomic fragment containing the first intron-exon junction and the eIF-2α promoter region was used with the same primer. B, six major transcription products indicated by arrows in panel A were mapped to the region of the Inr. The Inr element is identified by dots below the conserved bases.

Fig. 4. In vitro transcriptional analysis of wild type and mutant eIF-2α templates. In vitro transcription analysis of the wild type (wt) and mutant templates was performed as described for Fig. 2. Plasmids containing the eIF-2α promoter and sequences to +478 in intron 1 were digested with EcoRI and HindIII to release the 1.3-kilobase templates extending from −806 to +478. The 800-base pair XhoI-PstI fragment of the adenovirus 2 major late promoter was transcribed as a positive control. Lanes 2 and 6 contained α-amanitin (5 ng/ml). The size of the major run-off transcript from the wild type template is approximately 1250 nucleotides.
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In vitro transcription analysis was then used to ascertain transcriptional activity of the potential antisense Inr promoter. The results of in vitro transcription of a series of 5' Bal31 deletions of the promoter region of the eIF-2α gene are shown in Fig. 2. Transcription of the eIF-2α gene truncated at +478 was expected to yield a 478-nucleotide transcript whose abundance might reflect progressive deletion of promoter/enhancer/silencer elements. However, the major transcript from each template varied in size rather than in intensity. The size of the major transcript from each template was always 40-60 nucleotides less than that of the template and was shortened in proportion to the extent of the 5' deletion. This transcript overlapped the exon 1/intron 1 boundary of eIF-2α and was α-amanitin-sensitive (Fig. 2, panel C). These results strongly suggested the presence of an antisense transcription unit in the 3' portion of the in vitro eIF-2α transcription unit.

The 5' end of the in vitro antisense transcript was mapped by primer extension. Six sites were identified within a 40-bp region, and two of the start sites mapped to the consensus Inr element (Fig. 3). Mutation of the four core bases in the Inr element eliminated the in vitro transcript seen with the wild type construct (Fig. 4). In contrast to the in vivo CAT data, however, we were unable to see a sense transcript under the conditions used here even though the in vitro antisense transcript had been eliminated. In addition, mutation of three bases immediately downstream of the Inr element resulted in an approximately 2-fold decrease in the in vitro antisense transcript, a result which differs from our in vivo CAT data.

We next evaluated the ability of the region spanning the Inr element to drive a luciferase reporter gene when oriented in the antisense direction. Fig. 5 demonstrates that the sequences between +1093 and +179 are capable of generating moderate luciferase activity when compared with the promoterless vector. Deletion of the core three bases of the Inr element resulted in a reproducible 2.5-fold decrease in luciferase activity.

We have previously demonstrated that the primary transcript of the gene encoding eIF-2α is apparently destroyed in the nucleus when it is not needed and that during mitogenic stimulation of peripheral blood T cells, this transcript is stabilized long enough to be processed and transported to the cytoplasm (10). Regulation of the primary sense transcript by an overlapping antisense transcript is one mechanism by which such control might occur and has been proposed for a number of eukaryotic RNA polymerase II transcripts including c-erbAα, Xenopus basic fibroblast growth factor, and N-myc (24-26). Indeed, preliminary ribonuclease protection experiments in this laboratory have demonstrated the presence of a stable but weak antisense transcript mapping to this portion of the first intron in vivo in both quiescent and activated T cells.

Although no role in regulating sense transcripts has conclusively been proved for any of these antisense RNAs, alterations in RNA splicing or induction of a double-stranded RNA unwinding and modifying activity have been suggested as mechanisms by which these endogenous antisense RNAs might regulate expression of the sense transcript (24, 27). In the case of eIF-2α, the presence of such an antisense RNA in the first intron of the primary transcript might result in premature termination of transcription or transcriptional attenuation in G0, T cells. On the other hand, an antisense transcript that overlaps the splice site at the exon 1/intron 1 boundary might result in alternative splicing of the eIF-2α transcript and the formation of different eIF-2α mRNAs. In this regard, it is interesting to note that there are two hybridizing bands by Northern blot hybridization using both single- and double-stranded probes containing eIF-2α exonic sequences (10). The function of the downstream in vitro Dnase I footprint and the role played by an antisense RNA transcript in the regulation of eIF-2α expression as well as the role played by the two TATA-like elements and the Inr sequence in regulating expression of the antisense transcript are currently under investigation.

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