Regulation of mRNA abundance in activated T lymphocytes: Identification of mRNA species affected by the inhibition of protein synthesis

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ABSTRACT Inhibition of protein synthesis has often been observed to increase the concentration of mRNAs that encode proteins associated with the regulation of cell division. As two-dimensional gel electrophoresis permits the simultaneous monitoring of individual elements in large populations of gene products, we have used this technique to assess the effect of cycloheximide treatment on the mRNA complement of activated mouse T cells in an objective fashion. Two-dimensional gels of proteins generated by cell-free translation of mRNA from T-cell blasts display about 400 spots; only 5 of these are reproducibly enhanced by cycloheximide treatment and about 4 are diminished. The cDNA cloning vector λ jac allows analysis of large arrays of molecular clones by cell-free expression, and we have used it in a sibling selection scheme to isolate a clone of one of the prominently induced mRNA species, which we refer to as *chx1*. *chx1* mRNA concentration is increased by cycloheximide treatment of activated B cells, as well as T cells, and it is rapidly and transiently induced, in a cycloheximide-enhanced manner, upon serum stimulation of resting 3T3 fibroblastoid cells. The *chx1* protein is hydrophilic, is slightly basic, and has patches of homology with the *Jun-D* gene product. The *chx1* gene is remarkable in its lack of detectable introns and of strong bias against CpG dinucleotides.

The many thousands of mRNA species present in a cell occur at greatly different concentrations, determined for each species by rates of synthesis and degradation. There are multiple mechanisms that target mRNA molecules for destruction, and several of these may operate on transcripts of a single gene in an independent manner (1, 2), so that the lifetime of specific mRNAs can be subject to sensitive regulation. The seemingly wasteful balancing of high rates of synthesis and decay has a useful payoff in that it allows the concentration of some mRNAs to respond rapidly to altered environmental conditions. One might expect that mRNAs that encode proteins critical in regulating cell division should be prime candidates for such control, and indeed mRNAs for cytokines, oncogenes, and proteins associated with the G_0/G_1 transition tend to be short-lived (3-9). It has often been observed that this class of mRNA is greatly stabilized by inhibitors of protein synthesis, presumably because commitment to decay depends on a labile protein, because it depends directly on the process of translation, or both (3, 4, 6-9, 11, 12).

This information has generally emerged from experiments designed to assess the effect of an inhibitor or inducing agent on the concentration of one or several specific mRNAs within the cells of interest, by using probes already in hand. We wished, in essence, to reverse this information flow and to monitor mRNA concentration in a broad nonbiased study that would reveal species of mRNA responding to protein

synthesis inhibition, without prior structural information. Two-dimensional (2D) gel electrophoresis of proteins is appropriate to this task, as it has the capacity of detecting and quantifying thousands of different gene products simultaneously (13, 14). We have used the technique to compare mRNA preparations after translating them into polypeptides *in vitro*. When interesting translation products, such as the cycloheximide-induced mRNA we refer to as *chx1*, are identified in this fashion, the sequences encoding them* can be recovered from a cDNA library, by using a screen based on *in vitro* expression of recombinants of the positive selection vectors that this laboratory has developed (15, 16).

MATERIALS AND METHODS

Cells. Long-term activated T lymphocytes were elicited by culture of spleen cells from 8-week-old female BALB/cJ mice, suspended initially at 1×10^6 cells per ml in Iscove's modified Dulbecco's medium (IMDM) containing 10% (vol/vol) fetal calf serum and 50 μ M 2-mercaptoethanol, in the presence of concanavalin A at 2 μ g/ml for 85 hr at 37°C. Cycloheximide at 12 μ g/ml was added to some cultures at this time, and incubation continued for a further 3 hr. B-cell blasts were produced in similar cultures, replacing concanavalin A with a combination of bacterial lipopolysaccharide at 25 μ g/ml and dextran sulfate at 4 μ g/ml. T lymphocytes for short-term stimulation were purified from spleen cells by depletion of B cells and adherent cells on nylon wool columns and then activated by addition of phorbol myristate acetate and ionomycin (17) to 1 ng/ml and 100 ng/ml, respectively. NIH 3T3 cells were expanded in IMDM/10% fetal calf serum, starved for 2 days in IMDM/0.5% serum, and then stimulated with IMDM/20% fetal calf serum, in the presence or absence of cycloheximide at 12 μ g/ml.

Nucleic Acids, Molecular Cloning, and Cell-Free Expression. Preparative and analytical methods are described in detail elsewhere (14, 16, 18, 19). RNA was translated in nuclease-treated rabbit reticulocyte lysate (Amersham N90) in the presence of [35 S]methionine. Electrophoresis on 2D gels of translation products followed the protocol of O'Farrell (13) as modified by Anderson and Anderson (14). cDNA preparation and cloning in λ jac was as described (15, 16, 18, 19).

RESULTS

Comparison of mRNA Populations of Cycloheximide-Treated and Untreated T Cells by 2D Gel Electrophoresis of Cell-Free Translation Products. T lymphocytes in suspensions of mouse spleen cells were activated and expanded by addition of concanavalin A, then, after 85 hr of culture, samples were divided and cycloheximide was added to one aliquot. Cells were harvested from both aliquots after a further 3-hr culture period and mRNA was prepared from

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Abbreviation: 2D, two dimensional.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M31042).

both treated and untreated cells. Fig. 1*a* shows a representative 2D gel of proteins generated by translation of mRNA, in this case from untreated cells, in a nuclease-treated reticulocyte lysate. Comparison of such gels revealed that the vast majority of mRNA species detectable by this assay are unaffected by protein synthesis inhibition. However, visual inspection showed that five spots, labeled 1–5, were reproducibly enhanced among translation products of mRNA from cycloheximide-treated cells, whereas four, labeled a–d, appeared to be diminished by the inhibitor. The areas in which the altered spots occur are shown as frames i–v in Fig. 1*a* and isolated in Fig. 1*b*, which illustrates the effect of cycloheximide treatment. The reproducibility of this effect is demonstrated in Fig. 1*c*, which presents data for frame ii from two independent experiments. The enhancement of spots 1–5 is considerable and most striking for spots 1 and 2. Similar experiments (data not shown) indicated that spots 1 and 2 were also enhanced by cycloheximide treatment of lipopolysaccharide-stimulated B lymphocytes; these fluorographs were not sufficiently intense to allow visualization of the other species.

Molecular Cloning of *chx1*, a Cycloheximide-Enhanced mRNA Species. The observation that only 1–2% of detectable mRNAs were affected by cycloheximide immediately raised the issue of the identity of the altered species. Differential screening of cDNA libraries with probes from two mRNA populations has often been used to recover clones of differentially expressed genes, notably by Lau and Nathans (8, 9); however, a much more direct and precise approach was available from our development of cloning vectors that permit the characterization of large groups of molecular clones by the proteins that they encode (15, 16). λ jac is *spi⁺chi^o*, whereas recombinants of λ jac that contain cDNA sequences are *spi⁻chi⁺* and can be selected by growth on P2 lysogens of *Escherichia coli*. DNA from such recombinants can be transcribed with SP6 RNA polymerase to produce only sense-strand potentially translatable RNA, which we refer to as “ersatz mRNA.” Fig. 2 shows the electrophoretic profile of proteins produced by translation of ersatz mRNA from 17 sectors of a λ jac cDNA library from cycloheximide-treated T cells; each sector contained about 500 clones. On the same gel appear six tracks containing translation products

FIG. 2. Cell-free expression products of sectors of a cDNA library from cycloheximide-treated T blasts formed in the vector λ jac. Lanes + and – contained products of genuine mRNA isolated from T blasts, respectively, treated and untreated with cycloheximide, translated in reticulocyte lysate, and separated by standard PAGE in NaDodSO₄ on 10% polyacrylamide gels. Tracks 1–17 contain proteins translated from RNA transcripts (ersatz mRNA) of sectors of a λ jac cDNA library from cycloheximide-treated cells, each containing about 500 clones. Mobility of marker proteins of 97, 68, 43, 26, and 28 kDa is indicated at the side. The prominent common band at about 43 kDa is the endogenous reticulocyte component. Cycloheximide-enhanced species 1 is indicated with an asterisk.

of genuine mRNA from T cells, either treated or untreated with cycloheximide. These gels have poor resolution in the low molecular mass region, which is blurred by abundant mostly unlabeled globin, and cycloheximide-induced species 2 cannot be discerned; however, species 1 in Fig. 1 clearly shows up as a band with an apparent molecular mass of 33 kDa, visible only in the translation product of mRNA from treated cells. Preliminary analysis had suggested the presence of clones encoding this protein in six of the arbitrarily numbered sectors: these are grouped together at the left of

FIG. 1. Effect of cycloheximide on T-blast mRNA revealed by changes in the pattern of cell-free translation products. Cytoplasmic polyadenylated RNA was isolated from activated T cells and from similar cells treated for 3 hr with cycloheximide and then translated in a nuclease-treated reticulocyte lysate containing [³⁵S]methionine, and polypeptide products were displayed on 2D gels. (a) Fluorogram of a representative gel containing translation products of mRNA from cells untreated with cycloheximide. Regions containing spots affected by cycloheximide are framed and labeled i–v. The blurry vertically smeared complex to the right of frame i is due to nontranslational labeling of an endogenous reticulocyte component, and the blurry complex below frame iv is rabbit globin from residual endogenous mRNA. (b) Comparison of frames i–v from *a* (column –) with the same areas from a gel containing translation products of mRNA from cells treated with cycloheximide (column +). Species 1–5, enhanced by cycloheximide, and species a–d, diminished by cycloheximide, are indicated by arrows. (c) Effect of cycloheximide on frame ii, as seen in two independent experiments. Columns: +, cycloheximide treated; –, nontreated.

FIG. 6. Restriction enzyme map of the chromosomal *chx1* gene. Liver DNA was digested with restriction enzymes, electrophoresed through either a 0.7% standard agarose gel (a) or a composite gel of 0.8% standard agarose and 0.8% low-melting-point agarose (b), transferred to nitrocellulose, and hybridized with a labeled *chx1* probe. (a) Lanes: 1, BALB/cJ mouse DNA digested with *EcoRI*; 2, mouse, *HindIII*; 3, rat, *EcoRI*; 4, rat, *HindIII*. (b) Lanes: 1, mouse DNA was digested *HindIII*; 2, *HindIII* plus *SmaI*; 3, *HindIII* plus *BglII*; 4, *HindIII* plus *XbaI*. Above appears a map of cleavage sites in *chx1* cDNA, numbered from the 5' terminus of mRNA. H, *HindIII*; X, *XbaI*; B, *BglII*; S, *SmaI*. Mobility of DNA markers in nucleotides is given at the sides. An apparent absence of introns from the *chx1* gene is inferred from the similarity of restriction maps of the cDNA and the chromosomal gene.

SmaI within *chx1* cDNA and the Southern blot pattern produced by double digestion of mouse DNA with *HindIII* and one of the other three enzymes and by probing with *chx1*. The restriction enzyme analysis of mouse chromosomal DNA predicts a map of the *chx1* gene that agrees closely with that derived from *chx1* mRNA, within the limits of the Southern blot technique: introns are either entirely absent, or at most, occupy around a hundred nucleotides.

DISCUSSION

Cycloheximide Affects the Concentration of Few mRNAs in T Cells. Seemingly paradoxical inductive effects of inhibitors of macromolecular synthesis were noted by Tomkins *et al.* (20): they observed that hepatoma cells stimulated by dexamethasone greatly increased their rate of synthesis of tyrosine aminotransferase after addition of actinomycin D and attributed this to an increased stability of the mRNA encoding the enzyme. Similar superinduction by inhibitors of RNA or protein synthesis has been reported in many systems since then, notably of mRNAs encoding cytokines, cytokine receptors, some oncogene products, and proteins presumed to control the G_0/G_1 transition (3, 4, 6–9). We argued that, if enhancement by protein synthesis inhibitors is a common property of mRNAs specifying such a variety of critical regulators of cell growth, then reaction to this treatment might be a useful approach to identifying other mRNA species encoding regulatory proteins in T cells.

The display of cell-free translation products on 2D gels has been used surprisingly rarely to analyze complex mRNA populations (see ref. 21). Although the ratio of intensities of two spots in this system does not reflect the molar ratio of their respective mRNAs, relative changes in intensity of the same spots from two samples of mRNA can provide sensitive indications of relative changes in mRNA abundance, allowing us to use 2D gels of cell-free translation products to survey a large number of T-cell mRNAs simultaneously for

response to protein synthesis inhibition. The survey revealed that cycloheximide has a highly selective impact on mRNA abundance: of about 400 spots, only 5 were seen reproducibly to be increased in intensity, and 4 were reproducibly diminished.

The observation that cycloheximide specifically depletes some mRNAs underlines the complexity of the effects of inhibiting protein synthesis. Such inhibition directly affects the transcription of certain genes (6, 9, 22–24) as well as the lifetime of some mRNAs. Possibly the diminished species are short-lived mRNAs whose transcription depends on some labile protein.

***chx1* Protein.** The *chx1* protein is highly hydrophilic, which probably accounts for its anomalously slow electrophoretic migration in the presence of NaDodSO₄, due to sparse binding of the detergent: its true molecular mass is 24.5 kDa and apparent molecular mass is about 33 kDa. A search of the GenBank data base failed (at that time) to discover a consistent pattern of similarity between *chx1* and other known gene products. However, a visual scan of the *Jun-D* gene product, described by Nathans and coworkers (25), revealed some patches of similarity at the protein sequence level, shown in Fig. 7. *Jun-D* contains regions of strong nucleic acid homology with the two other members of the *Jun* gene family (26), which are less similar to *chx1*. Our attention was drawn to these genes because they are all induced, to different extents, in restimulated 3T3 cells and superinduced by cycloheximide (25, 27, 28). The three *Jun* proteins all appear to be sequence-specific DNA-binding proteins capable of forming complexes with the *fos* gene product (26), but, as the structure-function relationship of these proteins is not yet completely described, the significance of the patches of similarity with *Jun-D* exhibited by *chx1* is obscure.

We think that the *chx1* protein will turn out to be extremely labile (*i*) *a priori* because a stable protein could not play the fleeting regulatory role suggested by the rapidity of the appearance and disappearance of the mRNA and (*ii*) because the *chx1* protein is replete with stretches rich in proline, glutamate, and serine, considered to earmark short-lived proteins (29).

The *chx1* Gene. The doublet CpG has been long known to be markedly underrepresented in mammalian chromosomal DNA, presumably because spontaneous deamination of 5-methylcytosine, which occurs only in the doublet CpG, leads, over evolutionary time, to the decay of CpG to TpG, in the absence of other constraint. Table 1 indicates that CpG is underrepresented by a factor of about 4 in mouse DNA; however, its frequency in the *chx1* sequence, and also in the structural elements of the three *Jun* genes, approximates that expected from the individual occurrence of cytosine and guanosine in these sequences. CpG sequences are frequent in nonmethylated islands of DNA associated with transcriptional promoters of many genes (31), but the incidence of the doublet in the structural elements of most cloned genes, and in bulk cDNA from mouse embryo (G. Russell and C.C., unpublished observations) shows a deficiency similar to that of bulk nuclear DNA. An absence of such a deficiency in *chx1* and the *jun* genes would result from an absence of cytosine methylation within these genes in the germ line: CpG doublets that were never methylated in the germ line would not

#57 PesPFsrrALdPrl HPPReAevavE VaSpeaqPF epMDTQEvrlrvqEtpaLcdpppArvsRKRR #122
PfpPFg ALgPppppHPPIAalkdEpqtpvdpSfsgsPflspidMDTQERika ErkrLnriaAskcRKRR #270
#212

FIG. 7. Comparison of *chx1* and *Jun-D* protein sequences. The sequence of the predicted *chx1* protein from residues 57 to 122 is compared to that of the predicted mouse *Jun-D* protein (25) from residues 212 to 270. Identical residues are shown in bold capitals. Gaps are introduced to maximize similarity, but residues are otherwise contiguous. The single-letter amino acid code is used.

Table 1. Expected and observed frequencies of NpG dinucleotides

Dinucleotide	Frequency, %									
	Total mouse		chx1		c-jun		Jun-B		Jun-D	
	exp	obs	exp	obs	exp	obs	exp	obs	exp	obs
ApG	6.6	6.4	6.2	8.4	6.9	7.9	6.3	7.1	5.2	5.9
GpG	4.6	5.5	9.6	8.3	8.2	7.7	10.1	10.4	10.5	10.4
TpG	6.0	7.3	5.9	6.7	5.8	7.0	5.5	5.8	5.4	5.4
CpG	4.5	1.1	9.2	7.5	7.8	6.1	9.9	7.4	11.3	10.6
CpG _{exp} /										
CpG _{obs}	4.1		1.2		1.3		1.3		1.1	

Shown are frequencies of the four possible dinucleotides containing guanosine as the 3' member, expressed as percentage of all dinucleotides in the sequence. Expected (exp) frequencies are calculated by multiplying the frequencies of the single bases; observed (obs) frequencies are those that actually occur. Data for mouse chromosomal DNA in bulk is taken from ref. 30. Data for chx1 is from the sequence presented herein; data for the three mouse *jun* genes were obtained from the GenBank data base.

drift to TpG. Similar suggestions have been made to account for the frequency of CpG in promoter-associated methyl-free islands (31, 32). Although the relationship is not simple, there is a tendency for expressed genes to be undermethylated (33). Perhaps the inferred lack of germ-line methylation of chx1 and the *jun* genes reflects a continuing requirement for the products of these genes throughout gametogenesis. Some promoter-associated CpG islands are quite long, however, and can include exon sequences, so the high frequency of CpG throughout the length of the chx1 gene might be simply a fortuitous consequence of its small size.

More surprising is the apparent absence of introns from the chx1 gene. The exons of mammalian genes are typically interspersed with introns whose aggregate length exceeds that of the exons by a factor of 5–10, so even if introns do exist within the chx1 gene, that they should comprise less than 10% of the exon length would still be highly anomalous. Two classes of genes are known to lack introns in mammals: histones and α and β interferons; chx1 clearly falls into neither class. The necessity of a very rapid response to inducers would impose a constraint on the overall size of the chx1 gene. Transcription is a relatively slow process: as bacterial RNA polymerases achieve rates of chain-length elongation of 12–20 nucleotides per sec at 30°C on naked DNA (34), one might guess that it would take about 1 min to produce a 1-kb transcript in mammalian cells, and most direct estimates approximate this guess (35). We have not yet determined the time of initial appearance of chx1 mRNA in the cytoplasm of activated cells; however, its peak of accumulation probably occurs less than 30 min after serum addition, as its concentration is considerably greater at 30 min than it is 1 hr after stimulation. This would be impossible to achieve *de novo* from a gene containing long introns. We note that, although the *fos* gene, which also responds very rapidly to induction, does contain introns, they are short, comprising only 66% of the aggregate exon length in the human gene (36). Complete absence of introns from the chx1 gene could also greatly speed up the generation of functional mRNA from primary transcripts: although the appearance of significant amounts of labeled ribonucleoside in cytoplasmic poly(A)-containing RNA ensues 15–20 min after its addition to L cells, labeling of histone mRNA, whose synthesis requires neither polyadenylation nor removal of introns, proceeds with a much shorter lag (37, 38).

General Strategy for Recovering Cloned Genes Without Specific Probes. Sibling selection schemes, formally similar to

that used here to isolate chx1, have been employed a number of times to rescue genes encoding proteins with specific biological activities (10, 39). We have extended this approach by the use of purely physical criteria of clone selection, in the absence of any specific sequence information. This strategy should be applicable to any protein that can be physically identified by assays depending on its primary sequence. A data base of 2D protein gels, systematically matched with a sectorized cDNA library, should allow rapid identification and retrieval of clones specifying interesting proteins (15).

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