cate that the changes in heme-protein interactions produced by the optical triggers are insufficient to do this. These optical triggers do, however, provide a unique opportunity to investigate the nanosecond-microsecond dynamics of the unfolded protein prior to surmounting the free energy barrier that separates the unfolded structures from the native state (*9*).

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5. As with many different types of single-laser-pulse, initiated processes, reversibility of the reaction puts limits on the *longest* times that can be studied. For cytochrome c, reversal of the photochemistry aborts folding at a few milliseconds. In the electron transfer experiment, reoxidation of the reduced cytochrome by the Ru^{3+} complex occurs, while in the photodissociation experiment CO rebinds. Both of these problems could be overcome by using a continuous wave laser to maintain reduction or ligand dissociation after the initial excitation pulse.
6. The relaxation times corresponding to the rate constants obtained by Jones *et al.* (*2*) for the kinetic scheme in Fig. 1 are ~4 μs and ~120 μs. After reduction in the electron transfer experiment, the heme is mainly six-coordinate, and the same two relaxation times should be observed as in the CO photodissociation experiment. At most wavelengths, however, the amplitude will be dominated by the slower relaxation time corresponding mainly to histidine dissociation and rebinding. Considering the small difference in solution conditions (for example, the slightly higher pH in the experiments of Pascher *et al.*, which would result in less protonation of the histidines and faster binding rates) and the report of a single relaxation time in the electron-transfer experiment, we assume that the two techniques are giving consistent results, as also concluded by Pascher *et al.* (*4*). Thus, although the initial conditions are different in the two experiments, after ~100 μs they should produce the same distribution of denatured structures.
7. The quantum yield of fluorescence relative to the quantum yield in the absence of heme quenching is given by Forster theory as $[1 + (r_o/r)^6]^{-1}$, where r is the heme-tryptophan distance and r_o, which depends on the "overlap integral" of the normalized fluorescence spectrum of the tryptophan and the absorption spectrum of the heme, is ~3.5 nm. In a random coil distribution of heme-tryptophan distances, where the mean distance is ~7 nm the average relative quantum yield is 0.8, while at a distance of 2.0 nm the relative quantum yield is only 0.03. In the native conformation this distance is 1.0 nm.
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Response: In our report about the electron-transfer (ET)–initiated folding of ferrocytochrome c (cyt c^{II}) (*1*), we noted rapid changes in the visible absorption spectrum corresponding to a process with a time constant of ~40 μs. We indicated that this observation was consistent with studies of cyt c^{II} folding initiated by CO dissociation where the fast dynamics were attributed to changes in heme ligation (*2*). We also suggested that these dynamics might correspond to the collapse of the protein into a compact denatured state, as has been proposed for apomyoglobin on the basis of laser-temperature-jump measurements (*3*). Our transient absorption data could not distinguish between the two possibilities, and these fast folding dynamics were a relatively minor component of the study described in our report. Chan *et al.*, with the use of tryptophan (Trp) fluorescence as a probe, found no significant collapse of the protein on the submillisecond time scale following dissociation of CO from unfolded cyt c^{II}. Clearly, multiple spectroscopic probes must be employed to study protein folding; accordingly, we are currently developing time-resolved Trp fluorescence as a probe for ET-initiated folding of cyt c^{II}.

A particularly significant difference between our work and that of Chan *et al.* is the nature of the unfolded form of the protein. The initial state in ET-triggered cyt c^{II} folding is guanidine hydrochloride (GuHCl) denatured ferricytochrome c (cyt c^{III}) (*1*, *4*); in contrast, Chan *et al.* start with CO-ligated cyt c^{II} in the presence of GuHCl (*2*). Higher GuHCl concentrations are required to unfold CO-bound cyt c^{II} than cyt c^{III}. Apparently, the positive charge on the heme in cyt c^{III} has a significantly greater destabilizing effect than does CO binding to the ferroheme. Given these differences, it is reasonable to question whether the two unfolded states are the same. Investigations of cyt c^{III} folding using stopped-flow kinetic spectroscopy have been interpreted in terms of a minor collapse during the mixing dead time (~2 ms) (*5*). Nevertheless, the reduction in Trp fluorescence observed in this burst phase is measurably greater than the changes found by Chan *et al.*; this reduction has been attributed to a decrease in the Trp to heme distance of ~5 Å. Given the possible differences between the unfolded forms of the proteins, we must use Trp fluorescence as an additional probe in our studies of ET-initiated cyt c^{II} folding.

The complex process of protein folding is believed to involve dynamics that span more than 12 orders of magnitude in time (picoseconds to minutes). The power of optical triggering methods (such as photo-induced CO dissociation and ET chemistry) is that they lay open a large part of this time regime for direct examination.

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Complementary DNA for 12-Kilodalton B Cell Growth Factor: Misassigned

The 12-kD B cell growth factor (BCGF), also known as low molecular weight–BCGF or BCGF, is a cytokine produced by activated T lymphocytes (*1–4*). Several functions have been implicated for this factor. Most importantly, 12-kD BCGF has been suggested to be a common progression factor for human B lymphocytes and to have an autocrine role in B cell neoplasms (*5*). Twelve-kilodalton BCGF was purified to homogeneity in 1985 (*3*), and cloning of the corresponding cDNA was reported 2 years later (*4*). However, these studies have not been confirmed, and the exact molecular connection between the widely used commercial BCGF (purified from the supernatants of mitogen-activated lymphocytes), natural 12-kD BCGF, and the reported cDNA has remained ambiguous. Recently, a genomic segment that clearly corresponds to the reported cDNA sequence was identified (*6*, *7*). Sequencing of the genomic DNA of 12-kD BCGF by Zięţkiewicz *et al.* (*6*)

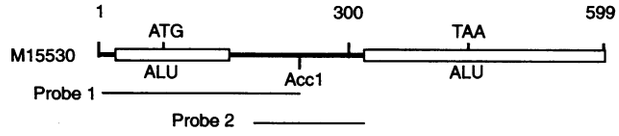

Fig. 1. Schematic presentation of the nucleotide probes used to detect 12-kD BCGF mRNA expression and their relation to ALU sequences present in M15530. Probe 1 was used by Sharma *et al.* (*4*) and probe 2 was used in this study. Open boxes represent ALU-derived sequences. ATG marks the start codon in M15530 ORF, and TAA the translation stop codon.

revealed four errors in the published (*4*) cDNA sequence, which resulted in a different open reading frame (ORF) within the 37 amino-terminal amino acids (*7*). An unusual, although not unique (*8*), feature in this sequence is the presence of ALU sequences that contribute to approximately two-thirds of the predicted protein. The ORF, when translated as a glutathione-S-transferase fusion protein in *Escherichia coli*, has B cell growth factor activity (*7*).

To study the expression pattern of 12-kD BCGF, we used the ALU-free portion of the nucleotide sequence to synthesise DNA and RNA probes (GenBank accession number M15530) (probe 2 in Fig. 1). The specificity of the probe for M15530 12-kD BCGF has been verified in Southern blots of genomic DNA (*7*). Unexpectedly, we did not detect any M15530 mRNA expression in mitogen-stimulated human peripheral blood lymphocytes (Fig. 2). The experimental procedures were similar to those reported by Sharma *et al.* (*4*, *9*). Peripheral blood mononuclear cells (PBMC) were stimulated for 18 hours with phytohemagglutinin (PHA), concanavalin A (Con A), or lipopolysaccharide (LPS). Total RNA was isolated from the cultured cells, run in formaldehyde-agarose gel, transferred onto nitrocellulose membrane, and hybridized to a randomly primed ALU-free fragment of M15530 sequence. The filter was washed in low-stringency conditions and exposed for autoradiography. A strong hybridization signal was detected with the positive control (1 ng of M15530 polyadenylated mRNA expressed under baculovirus polyhedrin promoter in Sf9 insect cells), but no signal was present in the lanes containing RNA from activated lymphocytes (Fig. 2A). The cells were properly activated as shown by the interleukin-2 receptor α-chain (IL-2Rα) mRNA expression (Fig. 2B). We also made significantly more sensitive ribonuclease (RNAase) protection analyses with similar results (*10*) (Fig. 3, A and B). Reverse transcription–polymerase chain reaction (PCR) analysis was not attempted because the genomic sequence could not be distinguished from the proposed cDNA sequence (*6*, *7*).

Our results show that mitogen-stimulated lymphocytes do not synthesize sufficient quantities of M15530 mRNA to be detected by Northern blot or by ribonuclease protection analyses. The apparent discrepancy with previous studies showing M15530 mRNA expression by activated T cells is most probably explained by the unusual features of the sequence, namely the presence of ALU-sequences. In the original cloning paper (*4*), a probe with approximately 50% of its nucleotides derived from ALU sequences was used for RNA analysis (probe 1 in Fig. 1). In our experiments, the same probe gave artifactual hybridization signals (data not shown). This can also be demonstrated by a BLAST-search (*11*) against the database of expressed sequence tags (dEST, National Center for Biotechnology Information) with the nucleotide sequence that was used as a probe in the original report. Such a search yields numerous highly homologous ALU-containing sequences from the dEST-database, but not one identical sequence when the database is searched with the ALU-free portion of the sequence (probe 2 in Fig. 1). Taken together, there is currently no evidence that would link the originally described 12-kD BCGF to the cloned cDNA, and we find that, for the present, 12-kD BCGF should be excluded from the continuously growing list of cloned cytokines.

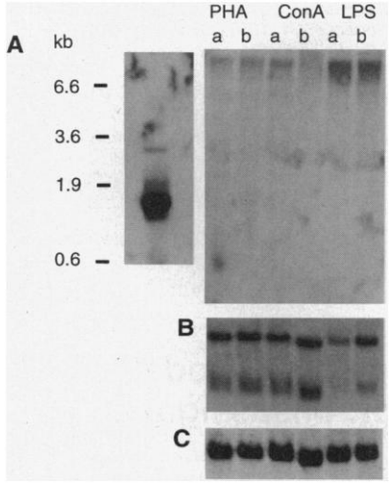

Fig. 2. Northern (RNA) blot analysis of 12-kD BCGF expression in mitogen-stimulated PBMC cells. (**A**) Total RNA (50 μg) from PHA-, Con A–, or LPS-activated PBMC cells of two donors (a and b) was hybridized to an ALU-free probe derived from the published sequence for 12-kD BCGF/M15530. First lane (outermost left) contains as a positive control 1 ng of polyadenylate RNA derived from Sf9 insect cells infected with M15530 mRNA-expressing baculovirus. (**B**) Same Northern blot hybridized to IL-2Rα probe and (**C**) to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. Molecular size standards are indicated at the left in kilobases.

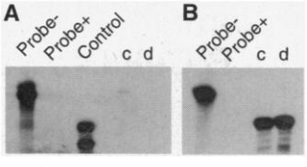

Fig. 3. Ribonuclease protection analysis of 12-kD BCGF mRNA expression in PHA-stimulated PBMC cells. Total cellular RNA (50 μg) isolated from PHA stimulated lymphocytes of two donors (c and d) was hybridized to ALU-free M15530 anti-sense RNA probe and analyzed on denaturing urea gels. (**A**) Full-length probe (probe –), ribonuclease-digested probe (probe +), 1 ng of Sf9 insect cells–derived RNA (control), and 50 μg of total RNA (c and d). (**B**) Five micrograms of total RNA from the same samples (c and d) were hybridized to β-actin anti-sense RNA probe.

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9. Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll-Isopaque density gradient centrifugation. Monocytes were not depleted. The cells (2 × 10^6 per milliliter) were suspended in culture media (RPMI 1640 supplemented with 10% fetal calf serum, glutamine, and antibiotics) and stimulated for 18 hours with PHA (2.5 μg/ml), Con A (5 μg/ml), or LPS (1 μg/ml) (all from Sigma Chemical Co., St.

Louis, MO). Total cellular RNA was isolated from the cells using the acid guanidium-isotiocyonate-phenol-chloroform-extraction meth → [P. Chomczynski and N. Sacchi, *Anal. Biochem.* **162**, 156 (1987)]. Fifty micrograms of total RNA was fractionated by agarose gel electrophoresis, blotted onto nitrocellulose filter, and hybridized to a randomly primed ^{32}P-labeled probe derived from the ALU-free portion of M15530 (nucleotides 112 to 240 from the ATG codon of the ORF, probe 2 in Fig. 2). The filter was washed in low-stringency conditions (3 × SSC and 0.1% SDS for 15 min at 65°C) and exposed to x-ray film at −70°C for 3 days. Polyadenylated RNA (1 ng) from Sf9 insect cells infected with M15530 expressing recombinant virus was included as a positive control. Afterward the filter was probed with human IL-2Rα probe and GAPDH probe.

10. Ribonuclease protection assays were carried out by using the RPA II ribonuclease protection assay kit according to the manufacturer's instructions (Ambion Inc., Austin, TX). The same 120-bp ALU-free insert of M15530 that was used for Northern analysis was cloned into pBluescript and used as a template for in vitro transcription. The 150-bp human β-actin control probe was purchased from Ambion. T7 polymerase was used to transcribe the anti-sense probes (Transcription in vitro system, Promega Inc., Madison, WI) in the presence of ^{32}P-labeled UTP. Forty thousand cpm of gel purified probes were used in overnight hybridization at 52°C with 50 μg (Fig. 3A) or 5 μg (Fig. 3B) of total RNA. The following day the samples were digested with ribonucleases A and T1 for 2 hours, then precipitated and fractionated in 6% urea gels. The gels were exposed to x-ray film for 12 hours at −70°C. Longer exposure (1 week) did not reveal specific signals.
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The findings of the mRNA analyses described above by Kovanen *et al.* (*1*) agree with our previous conclusion, based on phylogenetic analysis (*2*), that the reported M15530 cDNA (*3*) does not encode 12-kD BCGF. In their answer to our 1994 comment, Sharma *et al.* (*4*) proposed that the 12-kD BCGF was encoded by the originally reported cDNA, but in another reading frame starting from an AUG codon at residue 7 of the M15530 sequence. However, our phylogenetic comparison indicated that the newly proposed ORF not only had different lengths in all primates analyzed (121, 135, 59, 53, 71, and 71 amino acids in human, chimpanzee, gorilla, gibbon, baboon, and macaque, respectively) but also, as a result of frame shifts found only in certain species, differed in the amino acid composition. For example, the chimpanzee ORF was identical with the human for the first 101 codons, while in gorilla, gibbon, baboon, and macaque, a single A insertion changed the ORF after the first 45 codons in gorilla and gibbon, and after the first 47 in baboon and macaque. In the haploid human genome, a 120-codon-long ORF following an ATG codon would occur more than 10^5 times by chance alone, assuming an equal probability of all codons. Although our findings (*2*) do not formally eliminate the M15530 as 12-kDa BCGF coding sequence, they seriously call into question its functional nature, especially with no further evidence for the presence of transcriptional, translational, or RNA processing signals suggesting that the M15330 sequence was actively expressed.

Our criticism is supported by the work of Kovanen *et al.* (*1*). With the use of sensitive assays, they do not detect M15330-specific mRNA in cells known to express 12-kD BCGF. In agreement with these researchers, our search of the EST database (a total of 460,000 fragments of human mRNA sequences) using the BLASTN program resulted in no single relevant hit with their probe 2 sequence, and numerous (over 3000) hits with the Alu-containing probe 1 sequence. This is not unexpected because over 5% of human mRNAs contain an Alu sequence in their untranslated regions (*5*). Although Alu elements may in some cases provide the sequence for functional elements in proteins (*6*, *7*), the finding of Alu-related sequences in the coding regions of cDNAs raises the possibility of artifact and should be treated with caution (*8*).

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Response: The comment by Kovanen *et al.* accurately describes both the biological and molecular characteristics of a cDNA previously reported to putatively represent low-molecular-weight BCGF (12-kD BCGF) (*1*). The apparent verification of sequence by Southern analysis and confirmation of biological activity of the translated protein (*2*) substantiates several of the original observations, yet particular conundrums remain unresolved. The apparent inability of the Alu-free cDNA to hybridize to mRNA from activated human peripheral blood mononuclear cells brings forth the question of whether this cDNA accurately encodes the 12kD B cell proliferative activity previously isolated from normal activated human T lymphocytes and detected by distinct B cell proliferative assays. The sequence underlying the translation of an apparently functional B cell proliferative protein warrants investigation. However, it is evident that the presence of the Alu-encoding sequences may have led to the data originally compiled. For these reasons, we concur with the observations reported by Kovanen *et al.* and believe that the cDNA appropriately associated with 12-kD BCGF activity remains to be determined.

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