Putative Human Blue-Light Photoreceptors hCRY1 and hCRY2 Are Flavoproteins[†]

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ABSTRACT: Recently, a human cDNA clone with high sequence homology to the photolyase/blue-light photoreceptor family was identified. The putative protein encoded by this gene exhibited a strikingly high (48% identity) degree of homology to the *Drosophila melanogaster* (6−4) photolyase [Todo *et al.* (1996) *Science 272*, 109−112]. We have now identified a second human gene whose amino acid sequence displays 73% identity to the first one and have named the two genes *CRY1* and *CRY2*, respectively. The corresponding proteins hCRY1 and hCRY2 were purified and characterized as maltose-binding fusion proteins. Similar to other members of the photolyase/blue-light photoreceptor family, both proteins were found to contain FAD and a pterin cofactor. Like the plant blue-light photoreceptors, both hCRY1 and hCRY2 lacked photolyase activity on the cyclobutane pyrimidine dimer and the (6−4) photoproduct. We conclude that these newly discovered members of the photolyase/photoreceptor family are not photolyases and instead may function as blue-light photoreceptors in humans.

The photolyase/photoreceptor family of proteins are known or thought to mediate either DNA repair in many organisms or blue-light regulated developmental processes in plants by a photoinduced electron transfer reaction (Taylor, 1994; Menkens *et al.,* 1995; Heelis *et al.,* 1996; Sancar, 1996). Currently, this protein family is known to contain three members: cyclobutane pyrimidine dimer (Pyr<>Pyr) photolyase (Photolyase), (6−4) photolyase, and blue-light photoreceptor [see Todo *et al.* 1996]. The gene for the classical Pyr<>Pyr photolyase has been cloned, and the enzyme has been purified from many organisms including *Escherichia coli*, *Saccharomyces cerevisiae, Drosophila melanogaster*, and *Carassius auratus* (Sancar, 1990; Kato *et al.,* 1994; Yasui *et al.,* 1994). The (6−4) photolyase has been found in *D. melanogaster* (Todo *et al.,* 1993; Kim *et al.,* 1994), *Xenopus laevis*, and *Crotalus atrox* (Kim *et al.,* 1996b), but only the *Drosophila* gene has been cloned and sequenced (Todo *et al.,* 1996). The genes for the apoproteins of the blue-light photoreceptors of *Arabidopsis thaliana* (Ahmad and Cashmore, 1993), *Sinapis alba* (Batschauer, 1993; Malhotra *et al.,* 1995), and *Chlamydomonas reinhardtii* (Small *et al.,* 1995) have been cloned and sequenced, and the photoreceptors of *A. thaliana* (Malhotra *et al.,* 1995; Lin *et al.,* 1995b) and *S. alba* (Malhotra *et al.,* 1995) have been purified and characterized.

Recently, an analysis of partial cDNA sequences (expressed sequence tags) by Human Genome Science, Inc. revealed a tentative human consensus sequence homologous to the sequence of microbial photolyases (Adams *et al.,* 1995). The cDNA encompassing this sequence was isolated and sequence analysis of the entire open reading frame revealed a putative protein with high degree of sequence homology with the *D. melanogaster* (6−4) photolyase and extensive homology with the blue-light photoreceptors, followed by modest homology with the microbial type photolyases (Todo *et al.,* 1996). Thus, the possibility was raised that the "human (6−4) photolyase homolog" might function either as a repair enzyme or as a photoreceptor.

We wished to understand the function of this human (6−4) photolyase homolog by purifying the protein and testing it for enzymatic activities. During initial stages of our work, we identified a second gene which encoded for another (6−4) photolyase homolog. Both genes were expressed in *E. coli* and the encoded proteins, which we have named hCRY1 and hCRY2, were purified and characterized. We found that the human photolyase homologs, like other proteins in the photolyase/photoreceptor family, contain two chromophores: FAD and a pterin. The human photolyase homologs, the *D. melanogaster* Pyr<>Pyr photolyase and *D. melanogaster* (6−4) photolyase have nearly identical spectroscopic properties. Yet, when tested for repair, the *Drosophila* (6−4) photolyase repairs only (6−4) photoproduct, the regular photolyase repairs cyclobutane dimer exclusively, and the human photolyase homologs failed to repair either photoproduct. In addition, human cell-free extracts also lacked (6−4) photolyase activity. We conclude that the "human photolyase homologs" function as blue-light photoreceptors in a manner analogous to the plant blue-light photoreceptors.

MATERIALS AND METHODS

Clones for the Human Photoreceptors. The cDNA clone (R19031) for the human photolyase homolog (Adams *et al.,* 1995) carrying the 3′ terminal 1038 bp of the open reading frame gene was obtained from R. K. Wilson (Washington University, St. Louis, MO). The 5′ terminal part of the gene

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was obtained by using the 5′ RACE system for rapid amplification of cDNA ends (BRL) as described by the manufacturer using mRNA from a human fibroblast cell line (TO93). The amplified product was digested with *Nco*I and *Hind*III and cloned into the *Nco*I/*Hind*III sites of the baculovirus expression vector p2Bac (Invitrogen) and the *E. coli* expression vector pKK233-2 (Pharmacia). Sequence of the gene was confirmed by double-strand DNA sequencing using the Sequenase DNA sequencing kit (US Biochemical) and was in complete agreement with the previously published sequence (Todo *et al.*, 1996). A maltose-binding protein (MBP) fusion construct was made by inserting the BglII/*Hind*III fragment carrying the entire photolyase homolog coding region into the BamHI/*Hind*III site of the MBP expression vector pMal-c2 (NEB). This construct was named pDH1996-1.

The sequence of the second homolog was first identified by searching a database containing approximately one million human ESTs which was generated through the combined efforts of The Institute for Genomic Research and Human Genome Sciences Inc. using high-throughput automated DNA sequence analysis of randomly selected human cDNA clones (Adams *et al.*, 1991, 1992, 1995). Sequence homology comparisons of each EST were performed against the GenBank database using the blastn and tblastn algorithms (Altschul *et al.*, 1990). A specific homology search using the known human photolyase homolog 1 amino acid sequence against this human EST database revealed two ESTs (HGS6392 and HGS47815). Both were from a human fetal brain cDNA library, with greater than 84% homology to the first homolog. The two ESTs are identical except HGS47815 is 183 bp longer at the 5′ end. HGS47815 contains 3035 bp, and the sequence comparison suggested that it is missing approximately 1 kb of the putative photolyase homolog at the 5′ end. Using this clone as a probe, a hybridization screening was conducted through the human fetal brain cDNA library where HGS6392 and HGS47815 were initially discovered. From this screening, a positive clone (SO5), which was 466 bp longer than HGS47815, was identified.

To obtain the entire 5′ terminal part of the gene, the RACE PCR procedure was used. Briefly, a specific primer for the 3′ end of the gene, 5′-GGG CTC TGC CAC AGG GTG ACT GAG GTC-3′, was used for first strand cDNA synthesis. The first round of PCR amplification for the 5′ terminal part of the second photolyase homolog was carried out using a gene specific primer, 5′-AAT ACC CGG ACC CCG CTC-3′, at the 3′ end of the gene and a degenerative primer at the 5′ end as described by the manufacturer. This was followed by a second round of amplification using another gene specific primer, 5′-CAG GTC CCA CAG GCG GTA-3′, at the 3′ end of the gene and another degenerative primer at the 5′ end. Sequence comparison of the open reading frame of the amplified product to the first photolyase homolog confirmed that the 5′ end of the gene had been cloned.

An MBP fusion of the SO5 clone was constructed by ligating an *Eco*RI/*Bgl*II fragment containing the entire open reading frame of SO5 into the *Eco*RI/*Bam*HI site of pMal-c2. This construct, which contained the carboxy terminal 381 amino acids of the second photolyase homolog, was named pDH1996-2.

Purification of Recombinant Human CRY Proteins. Since our work indicates that the human photolyase homologs are not repair enzymes, but most likely blue-light photoreceptors (cryptochrome, CRY), we will refer to the genes and proteins as *CRY1* and *CRY2* and hCRY1 and hCRY2, respectively. Attempts to overproduce these proteins in *E. coli* as nonfusion forms failed. Therefore, MBP fusions of CRY1 and CRY2 were constructed and the proteins were purified by amylose affinity chromatography as described previously (Malhotra *et al*., 1995).

Spectroscopic Analysis. The absorption spectra of the purified proteins were recorded with a Hewlett-Packard model 8451A spectrophotometer, and the fluorescence spectra of the chromophores were measured at 22 °C in a Shimadzu RF5000 U spectrofluorimeter.

Photolyase Assay. This assay measures the restoration of the susceptibility to cleavage of the TTAA sequence by the *Mse*I restriction endonuclease in a DNA fragment where the TT is either in the form of a cyclobutane thymine dimer or (6−4) photoproduct (Malhotra *et al.*, 1995; Kim *et al.*, 1996b). A 54 mer and 49 mer duplex containing a centrally located T<>T and T[6−4]T, respectively, were prepared as described previously (Smith & Taylor, 1993) and were kindly provided by Dr. J.-S. Taylor (Washington University).

For the photoreactivation assay, proteins (40 nM) were mixed with 0.5 nM substrate in a 50 μL reaction containing 50 mM Tris, pH 7.4, 100 mM NaCl, 6 mM dithiothretiol, 2 mM EDTA, 5 μg of bovine serum albumin, and 5% glycerol. The mixture was incubated in the dark at room temperature for 10 min and then exposed to photoreactivating light (λ_{max} = 366 nm) at 4 °C for 1 h from a Sylvania black light (model B-100) at a fluence rate of 2 mW/cm^2. The DNA was then extracted with phenol/chloroform, precipitated with ethanol, resuspended in restriction enzyme buffer, and digested with 8 units of *MseI* for 1 h. The reaction products were electrophoresed on an 8% denaturing gel, and the level of digested (repaired) DNA was determined by a PhosphoImager (Molecular Dynamics Inc.).

Assays with human cell-free extract (CFE) were performed in a similar manner except 50 μg of CFE was used in the reaction.

RESULTS

Two Human Photolyase Homologs. Large-scale sequencing of expression sequence tagged (EST) cDNAs revealed a clone with homology to the microbial photolyase genes; this clone was designated a photolyase isolog since there is no convincing evidence that humans have a photolyase which can repair cyclobutane pyrimidine dimers (Adams *et al.*, 1995). Independently, Todo *et al*. (1996), cloned and sequenced the gene for the apoenzyme of the newly discovered (6−4) photolyase from *D. melanogaster*. It was found that the (6−4) photolyase has a high degree of homology with the photolyase/blue-light photoreceptor family of proteins (Ahmad & Cashmore, 1993; Malhotra *et al.*, 1995), including the human photolyase isolog. In fact, when the entire cDNA of the human photolyase isolog was isolated and sequenced it revealed an astonishing 48% sequence identity with the *D. melanogaster* (6−4) photolyase (Todo *et al.*, 1996).

Here we report isolation of a second human photolyase isolog. The second gene was originially identified in a

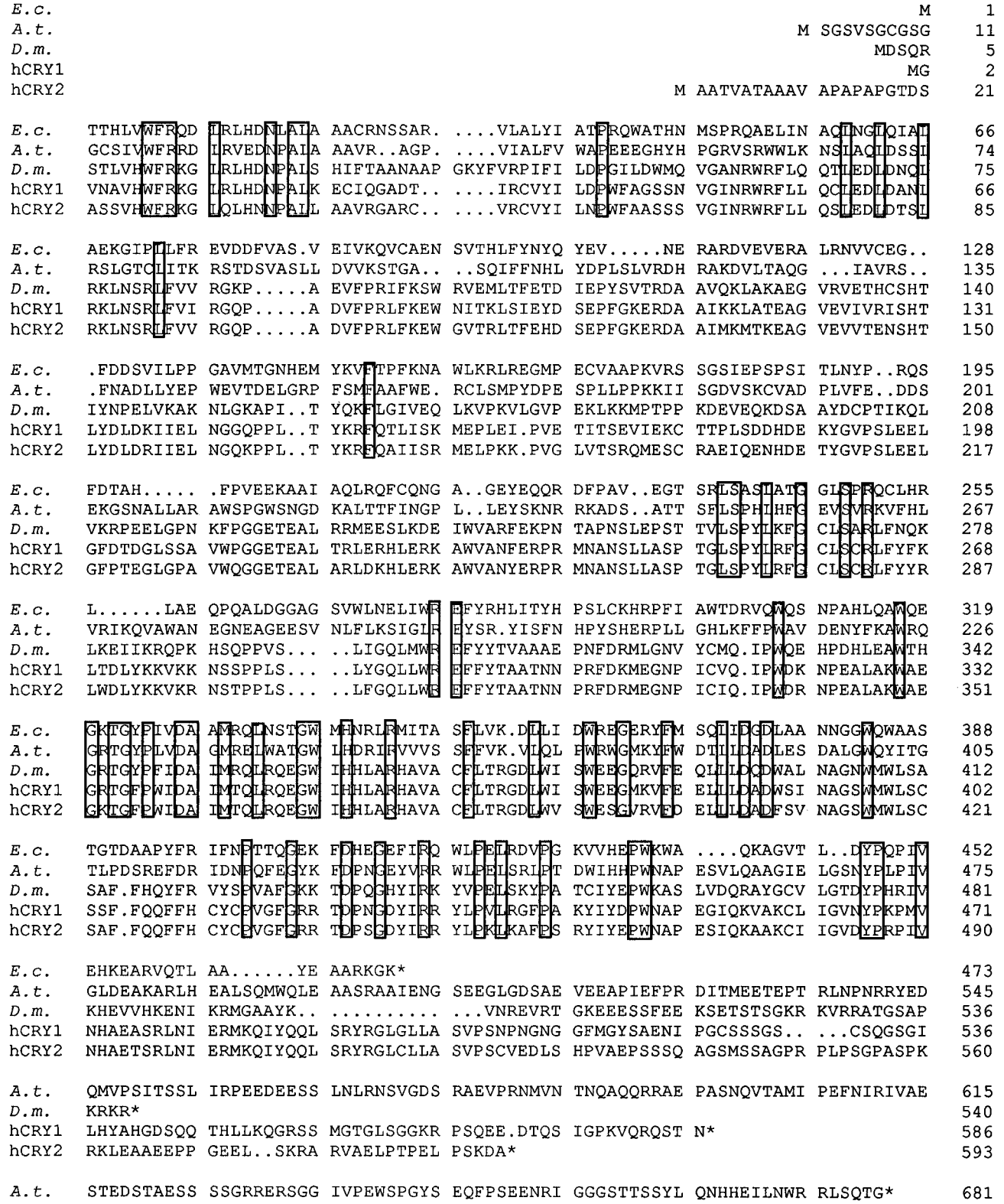

FIGURE 1: Sequence comparison of *E. coli* photolyase (*E.c.*), *Arabodopsis* HY4 photoreceptor (*A.t.*), *D. melanogaster* (6−4) photolyase (*D.M.*), and the putative human blue-light photoreceptors hCR1 and hCRY2. Amino acid residues which are identical in the entire set are boxed.

human fetal brain cDNA libray and was found to be expressed in fibroblasts as well. Of special note, the second isolog occurred at lower frequency in the human cDNA database compared to the first isolog in all tissues tested. Since the work that we will present below suggests that these photolyase isologs are more likely human blue-light photoreceptors, we will designate the gene identified previously (Adams *et al.*, 1995; Todo *et al.*, 1996) as *CRY1* and the newly identified gene *CRY2* to comply with the nomenclature for blue-light photoreceptors (cryptochrome) in plants (Short & Briggs, 1994). Sequence comparison of these two genes along with a representative member of a type I (microbial) class photolyase, the blue-light photoreceptor gene HY4 of *A. thaliana*, and the (6−4) photolyase of *D. melanogaster* are shown in Figure 1. The two human genes have 65% sequence identity at the nucleotide level and 73% sequence

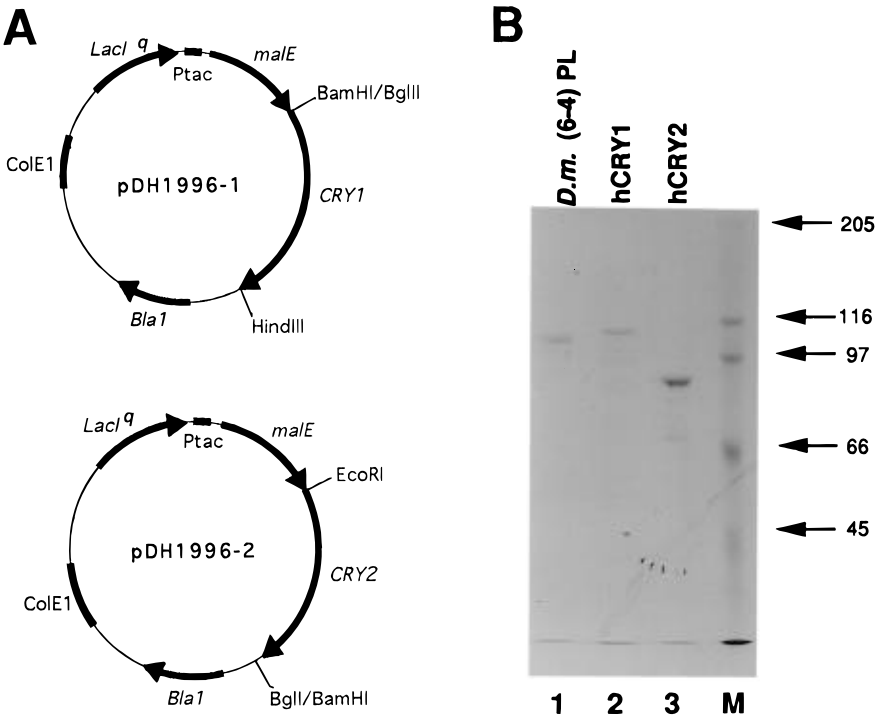

FIGURE 2: (A) Map of plasmids pDH1996-1 and pDH1996-2 for overexpressing hCRY1 and hCRY2, respectively, as maltose binding fusion proteins. The arrows indicate the length and direction of transcription of MBP (*malE*), *Bla*1, and photoreceptor genes. (B) Fusion proteins used in analysis of function of human photoreceptor genes. All proteins were in the form of MBP fusion. Lane M contains molecular mass markers in kilodaltons. The proteins were separated on a 8% SDS−PAGE and stained with Coomassie Blue.

identity at the amino acid level. The second gene also shows a high degree of sequence homology to *D. melanogaster* (6−4) photolyase with a 51% sequence identity over the entire length.

Aside from the high degree of sequence homology between hCRY1 and hCRY2, the most noteworthy feature of the sequences of these proteins is the complete divergence over the carboxy-terminal 80 amino acids. A similar feature has been found between the two *A. thaliana* blue-light photoreceptors (Ahmad & Cashmore, 1996), and since evidence exists that this "tail" region of the photoreceptor interacts with the effector molecule (Lin *et al.*, 1996a), it is conceivable that the two human proteins also interact with different downstream targets.

Purification of Recombinant Human Photoreceptors. To find out whether hCRY1 and hCRY2 are photolyases or photoreceptors, we purified these proteins and tested them only for photolyase activity since at present, we have no assay for a human blue-light photoreceptor. The proteins were expressed as fusion proteins using the MBP fusion vector pMal-c2 (Figure 2A) and purified by affinity chromatography on amylose resin. Since the possibility exists that fusion with MBP may interfere with enzymatic function, as a control we used an MBP fusion form of the *D. melanogaster* (6−4) photolyase (Todo *et al.*, 1996). This enzyme is highly homologous to the putative human photoreceptors and was purified in a similar manner. Figure 2B shows the three fusion proteins used in our spectroscopic and enzymatic analyses.

Spectroscopic Properties of hCRY1 and hCRY2. All photolyases and blue-light photoreceptors that have been characterized contain FAD and a second chromophore which is a folate in most organisms; in a few species which can synthesize deazaflavin, the second photolyase chromophore is deazariboflavin (Eker *et al.*, 1990; Malhotra *et al.*, 1995). We wished to identify the cofactors of hCRY1 and hCRY2 to determine if the photolyase sequence homology was accompanied by structural similarities. The absorption spectra of the MBP fusion forms of hCRY1 and hCRY2 are shown in Figure 3A. Both proteins have a distinct 420 nm peak with residual absorption extending all the way to 700 nm, and the absoprtion spectra are almost identical to the absorption spectra of the cyclobutane pyrimidine dimer photolyase (Kim *et al.*, 1996a) and the (6−4) photolyase (data not shown) from *D. melanogaster*. It has been demonstrated that the *D. melanogaster* T<>T photolyase contained FAD and folate as chromophores (Kim *et al.*, 1996a). Hence, we reasoned that hCRY1 and hCRY2 may also contain these cofactors. A simple assay revealed that this is indeed the case. hCRY1 and hCRY2 were denatured by heating for 10 min at 65 °C in 0.1 M HCl and 0.8% SDS. Following centrifugation to remove the protein precipitate, excitation and emission fluorescence spectra were recorded. Figure 3B shows a diagnostic flavin fluorescence spectrum (Faeder & Sieger, 1973); hence we conclude that both proteins contain flavin. Furthermore, when the pH was increased to 10 by addition of NaOH, the flavin fluorescence was severely quenched, further confirming the cofactor as FAD (Faeder & Siegel, 1973). Alkaline pH had another notable effect on the fluorescence spectrum: a new species with an excitation maximum at 380 and emission maximum at 470 appeared (Figure 3C). This behavior is typical of reduced pterin which is non-fluorescent but is converted to highly fluorescent oxidized pterin upon incubation in alkaline solutions (Johnson *et al.*, 1988). Furthermore, the excitation and emission spectra of the second chromophore are identical to that of the *D. melanogaster* T<>T photolyase which was shown to be a folate by TLC analysis with appropriate standards (Kim *et al.*, 1996a). Thus, we conclude that hCRY1 and hCRY2, like the majority of the members of

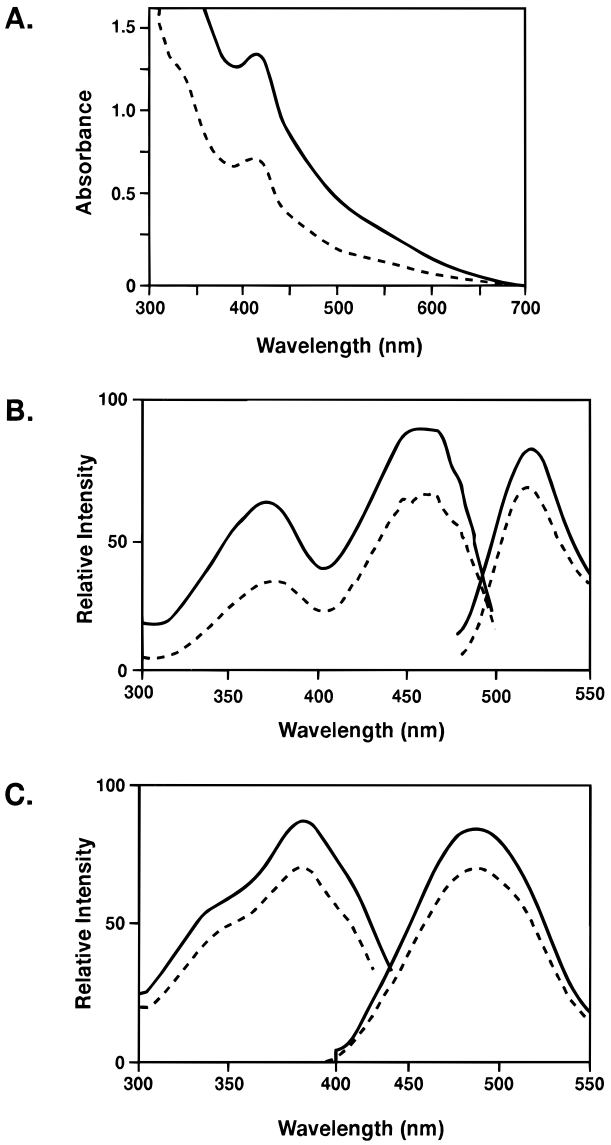

FIGURE 3: Absorption and fluorescence spectra of hCRY1 and hCRY2 proteins. The dashed line represents the spectra of hCRY1 and the solid line represents the spectra of hCRY2. (A) absorption spectra. (B) Fluorescence excitation and emission spectra of the hCRY1 and hCRY2 chromophores at pH 2. Fluorescence excitation spectra were recorded by monitoring emmision at 520 nm while fluorsence emmision spectra were recorded by using excitation at 450 nm. (C) Fluorescence excitation and emission spectra of hCRY1 and hCRY2 chromophores at pH 10. Fluorescence excitation spectra were recorded by monitoring emmision at 470 nm, while fluorsence emmision spectra were recorded by using 380 nm excitation.

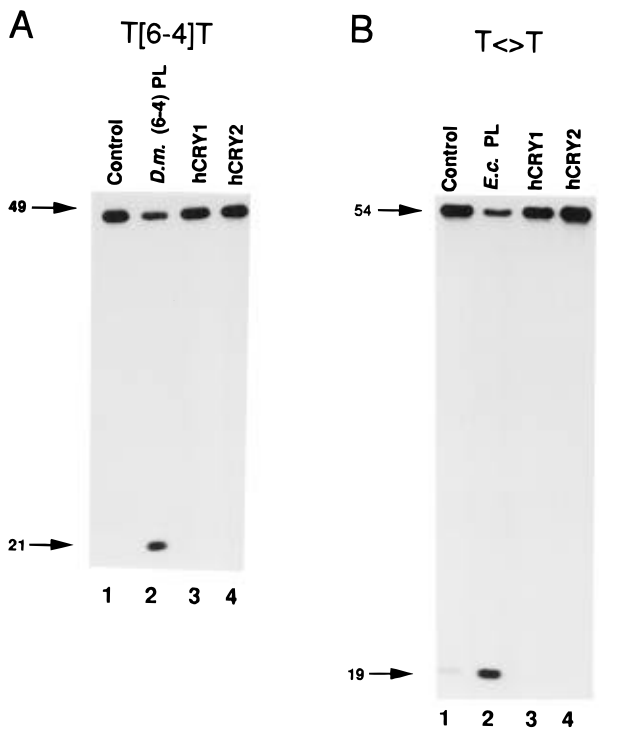

FIGURE 4: Photoreactivation assays with hCRY1 and hCRY2 proteins. A 50 μL reaction mixture containing substrate (0.5 nM) and protein (40 nM) was incubated in the dark at room temperature for 10 min and then exposed to photoreactivating light (λ_{max} = 366 nm) at 4 °C for 1 h. The DNA was then extracted, digested with *Mse*I and electrophoresed on an 8% polyacrylamid denaturing gel. For the (6−4) photoproduct and T<>T substrate, the levels of 21 mer and 19 mer, respectively, indicate the extent of repair. (A) Repair of T[6−4]T photoproduct. In lane 2, 48% of the substrate was repaired. (B) Repair of T<>T photoproduct. The 19 mer in the control lane is due to the presence of low levels substrate without the photolesion. This was substracted from the other lanes in calculating the level of repair. In lane 2, 54% of the substrate was repaired.

the photolyase/photoreceptor family, contains FAD and a pterin as the two chromophore/cofactors.

Lack of Photolyase Activity in hCRY1 and hCRY2. The spectroscopic properties of hCRY1 and hCRY2 are consistent with these proteins being a Pyr<>Pyr photolyase, a (6−4) photolyase, or a photoreceptor. To differentiate between these possibilities we tested the recombinant proteins for repair activity. Figure 4A and 4B shows that hCRY1 and hCRY2 failed to reverse either T<>T or T[6−4]T, while the *E. coli* photolyase and the *D. melanogaster* (6−4) photolyase, which have sequence and spectroscopic properties similar to the human proteins (Todo *et al.*, 1996; unpublished observation), repair T<>T and T[6−4]T, respectively. Conducting the repair experiments under a variety of conditions (higher protein concentration and higher dose of photoreactivating light) led us to conclude that hCRY1 and hCRY2 cannot have more than 0.1% of the photolyase activities detected with *bona fide* photolyases. It is unlikely that the lack of activity is due to fusion with MBP because the (6−4) photolyase which is used as positive control is also in the form of a MBP fusion and has spectroscopic properties identical to hCRY1 and hCRY2 (data not shown). Similarly, several photolyases including *E. coli* and *D. melanogaster* photolyases are fully active as MBP fusion proteins (Kim *et al.*, 1996a). Thus, we conclude that the recombinant photolyase homologs do not have photolyase activity.

Even though these data strongly suggest that hCRY1 and hCRY2 are not photolyases, it was conceivable that the proteins expressed in heterologous system were somewhat misfolded or lacked a posttranslational modification necessary for activity. Hence we tested the natural source for activity. Cell-free extracts from fibroblasts (TO93), which expressd hCRY1 and hCRY2 as revealed by primer extension (for both hCRY1 and hCRY2) and immunoblotting (for hCRY1 only), failed to show any (6−4) photolyase activity (Figure 5). To ascertain that this lack of activity was not due to inhibition by other proteins known to exist in CFE which bind to (6−4) photoproduct (Chu & Chang, 1988; Ghosh *et al.*, 1996; Wakasugi *et al.*, 1996), we mixed *Drosophila* (6−4) photolyase with the fibroblast cell-free

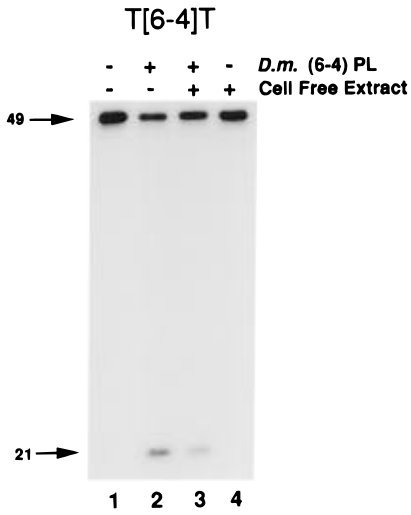

FIGURE 5: Photoreactivation assay with cell-free extract. A 100 μL reaction mixture containing substrate (0.5 nM) and cell-free extract (50 μg) was incubated in the dark at room temperature for 10 min and then exposed to photoreactivating light (λ_{max} = 366 nm) at 4 °C for 1 h. The DNA was extracted, digested with *Mse*I, and separated on an 8% polyacrylamide gel. The level of 21 mer indicates the extent of repair. In lane 2, 38% of the substrate was repaired, while in lane 3, 29% of the substrate was repaired.

extract and conducted photoreactivation with this mixture. A slight inhibition of *Drosophila* (6−4) photolyase was observed in the presence of CFE, but the level of inhibition cannot explain the total lack of activity in CFE under a variety of conditions. Finally, hCRY1 purified from a baculovirus/insect cell expression system also failed to show any photolyase activity (data not shown). Taken together, our data suggest that hCRY1 and hCRY2 are not photolyases and more likely function as blue-light photoreceptors.

DISCUSSION

Many attempts from several labs to detect and isolate photolyases from human cells failed, leading to a near-consensus in the field that humans do not have photolyase (Ley, 1993; Li *et al.*, 1993; Kato *et al.*, 1994). However, the recent discovery of a photolyase for (6−4) photoproducts and the finding that it belongs in the photolyase/photoreceptor family of proteins (Todo *et al.*, 1996) raised the interesting possibility that humans might have a (6−4) photolyase. Furthermore, the identification of a human homolog with 48% sequence identity with *D. melanogaster* (6−4) photolyase (Todo *et al.*, 1996) made such a possibility quite likely. The encoded protein could be the elusive cyclobutane pyrimidine dimer photolyase (Sutherland & Bennett, 1995), a (6−4) photolyase which had not been searched for in humans in a systematic way, or a photoreceptor. This work was undertaken to differentiate between these possibilities.

Our results clearly show that the human (6−4) photolyase homolog identified previously, and the new homolog we have identified is neither (6−4) photolyase nor cyclobutane pyrimidine dimer photolyase. Work with recombinant proteins and human cell-free extract showed that the proteins encoded by these two genes neither bind to UV-damaged DNA (data not shown) nor repair T<>T or T[6−4]T photoproducts in the absence or presence of light. Thus, we are left with the third alternative, that the proteins encoded by these two photolyase isolog genes function as blue-light photoreceptors.

Currently, most of the work on blue-light photoreceptors is being conducted with plants (Hohl *et al.*, 1992; Short & Briggs, 1994; Hinnemann, 1995) and fungi (Dunlap, 1993). In plants, blue light induces responses such as photomorphogenesis, phototropism, and hypocotyl elongation. In particular, it has been demonstrated that the HY4 gene of *A. thaliana* which encodes CRY1 is required for blue-light induced hypocotyl elongation (Ahmad & Cashmore, 1993). In animals, most of the work on light response (other than vision) has been concentrated on the circadian clocks. In *D. melanogaster* two genes, *timeless* and *period*, which regulate the circadian rhythm have been cloned (Myers *et al.*, 1995; Gekakis *et al.*, 1995). Both appear to be transcription factors whose activity is regulated by light. A mutation in a gene (*tau*) which disrupts the circadian clock was identified in the golden hamster several years ago (Ralph & Menaker, 1988) and three genes, *CLOCK*, *ICER* and *CREM*, which are involved in the control of circadian rhythm in mouse have been investigated in some detail (Vitaterna *et al.*, 1994; Sassone-Corsi, 1995; Foulkes *et al.*, 1996). All three gene products appear to be transcriptional repressors, and their activities are regulated by light. However, how the light signal is transmitted to the transcriptional regulators is not known. We believe that further studies on human *CRY1* and *CRY2* and their rodent homologs will help answer some of these questions.

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