Mouse mCRY1 and zebrafish zCRY1a and zCRY3 belong to the DNA photolyase/Cryptochrome family. mCRY1 and zCRY1a repress CLOCK:BMAL1-mediated transcription, whereas zCRY3 does not. Reciprocal chimeras between zCRY1a and zCRY3 were generated to determine the zCRY1a regions responsible for nuclear translocation, interaction with the CLOCK:BMAL1 heterodimer, and repression of CLOCK:BMAL1-mediated transcription. Three regions, RD-2a-(126–196), RD-1-(197–263), and RD-2b-(264–293), were identified. Proteins in this family consist of an N-terminal α/β domain and a C-terminal helical domain connected by an interdomain loop. RD-2a is within this loop, RD-1 is at the N-terminal 50 amino acids, and RD-2b at the following 31 amino acid residues of the helical domain. Either RD-2a or RD-1 is required for interaction with the CLOCK:BMAL1 heterodimer, and either RD-1 or RD-2b is required for the nuclear translocation of CRY. Both of these functions are prerequisites for the transcriptional repressor activity. The functional nuclear localizing signal in the RD-2b region also was identified. The sequence is well conserved among repressor-type CRYs, including mCRY1. Mutations in the nuclear localizing signal of mCRY1 reduce the extent of its nuclear localization. These findings show that both nuclear localization and interaction with the CLOCK:BMAL1 heterodimer are essential for transcriptional repression by CRY.

Organisms ranging from bacteria to humans have daily rhythms driven by endogenous oscillators called circadian clocks that regulate various biochemical, physiological, and behavioral processes with a periodicity of approximate by 24 h (1–3). Under natural conditions, rhythms are entrained to a 24-h day by environmental time cues, most commonly light. These circadian clock mechanisms have been investigated by characterizing the “clock genes” that affect the daily rhythm. The core of the clock mechanisms in Drosophila, Neurospora, mammals, and cyanobacteria is expressed by a transcription/translation-based negative feedback loop that relies on positive and negative oscillator elements. The negative feedback loop begins by activating the transcription of clock genes, the products of which then negatively regulate their own expression, setting up the rhythmic oscillations of gene expression that drive the circadian clock. Although negative feedback loop is a common mechanism in Drosophila, Neurospora, mammals, and cyanobacteria, its components differ with the species. Drosophila and mammals have common components (orthologous gene products), except for the negative elements TIM1 (TIMELESS) and CRY (Cryptochrome). PER (PERIOD), and TIM, identified as the negative elements in Drosophila, form a heterodimer that translocates to the nucleus where its components interact with the positive elements dCLOCK and CYC (4). Formation of a complex decreases dCLOCK:CYC-mediated transcription, resulting in the repression of expression (5, 6). In mammals, CRY1 and CRY2 are partners of the PER heterodimer, rather than TIM. In mammals, the mPER and mCRY proteins form heterodimers that translocate to the nucleus where they act as negative regulators by interacting with CLOCK and BMAL1 (CYC is the Drosophila homolog of BMAL1) to inhibit transcription (7, 8). In Drosophila a different role has been identified for dCRY (9–11). It binds TIM and PER in a light-dependent manner (12, 13). This interaction disrupts their inhibitory effect on transactivation of the CLOCCYC heterodimer, leading to a phase shift in circadian rhythm. Consistent with this model, cry2 mutant flies that bear a mutation within the dcry gene display free-running behavioral rhythms but lack light entrainment capability. Furthermore, these flies show rhythmicity in constant light, whereas wild-type ones are arrhythmic under such conditions (9). These findings show that the role of dCRY is as a circadian photoreceptor and indicate diversity in CRY functions in different species. Mouse CRY was designated a repressor-type, Drosophila CRY a non-repressor-type.

The zebrafish provides an attractive vertebrate model for biological clock analyses. Several of its clock genes have now been identified, and in vitro analyses has shown that the zebrafish negative feedback loop consists of components similar to those of mammals. zPER and zCRY act as negative regulators, and zCLOCK and zBMAL as positive elements (14–20). Four repressor-type zCRYs (zCRY1a, -1b, -2a, and -2b) have been identified in zebrafish. They repress the activities of mouse CLOCK and BMAL, as well as those of zebrafish CLOCK and BMAL, indicative that the basic function of repressor-type.
pressor-type cCRYs is the same as that of mCRYs (16, 19, 20). A unique feature of zebrafish CRY is the presence of extra pressor-type cCRYs is the same as that of mCRYs (16, 19, 20).

Expression System—Novel restriction enzyme sites were introduced within zCRY1a and -3 to prepare the zCRY1a-3 chimeras. Site-directed mutagenesis system Mutan-Super Express KM kit (Takara) was used, which included a PmaCI site at bp 375 of the cDNA encoding zCRY1a, and PmaCI at bp 375, SacI at bp 589, SalI at bp 785, and EcoRI at bp 880 of the nucleotide sequence encoding zCRY1. Synthetic oligonucleotides were used for mutagenesis: for PmaCI in the zCRY1a sequence, 5'-GGAGGTTGATAGCTGACTCCTACA-3' (covering bp 386–389); for PmaCI in the zCRY3 sequence, 5'-GGAAACCTAGTCTAACCCTGACA-3' (covering bp 366–390); for SacI in the zCRY3 sequence, 5'-CTTCUTCAAGGAGTCCGGTTAGG-3' (covering bp 589–603); for SalI in the zCRY3 sequence, 5'-TGCATCTCATGCTGTTAATCTGA-3' (covering bp 789–800); and for EcoRI in the zCRY3 sequence, 5'-GGGGAGAAATCTTACACG-3' (covering bp 875–894). Each of these oligonucleotides is underlined above. The zCRY1a-3 chimeras shown in Figs. 2A and 3A were generated by switching each segment between zCRY1a and -3 at the newly introduced restriction enzyme sites, the EcoRI site of the mature CRY3, and the EcoRI site of the mCRY1 as a cofactor to maintain proper conformation, and FAD bind-
RESULTS

Construction of Chimeric zCRYs with Exchanged zCRY1a and zCRY3 Regions—A comparison of the predicted amino acid sequences of mCRY1 and zCRY1a and -3 shows that the zebrapine fish genes are close structural homologs of mCRY1 (Fig. 1A). CRY protein consists of the N-terminal chromophore-binding and C-terminal extension domains, the former well conserved among all the CRY proteins, the latter having no known homology (22, 23, 29). In fact, the sequence of the former domain is highly conserved between mCRY and zCRY1a and -3 with 77–81% identity (Fig. 1A). Despite sharing a well conserved primary structure, the three CRYs differ markedly in their functional activities. zCRY1a, as well as mCRY1, represses CLOCK:BMAL-mediated transcription, whereas zCRY3 does not (16, 20). We took advantage of the similarities between the nucleotide/mino acid sequences and the differences in transcriptional repressor activities of zCRY1a and -3 to produce zCRY1a-3 chimeras, which permitted mapping of the region responsible for transcriptional repressor activity. Alignment of the amino acid sequences of zCRY1a and -3 showed no insertion or deletion of the amino acid sequences between the N-terminal chromophore-binding domains of the two proteins (Fig. 1A), indicative of minimized disruption of the native conformation by chimera construction. To generate the chimeric constructs, restriction recognition sites were created in each cDNA clone by site-directed mutagenesis. In each mutagenesis, alterations in the nucleotide sequences were designed to minimize changes in the encoded amino acids. To determine which domain to exchange reciprocally, we constructed a structural model of zCRY1a, using the crystal structure of E. coli photolase, a member of the DNA photolysis/Cryptochrome family, as the starting point (Fig. 1B). With this structural model as a guide and considering restrictions within the nucleotide sequence for creating new restriction enzyme sites, six zCRY1a regions were selected for reciprocal domain (A), structural model (B), and schematic representation (C). A, sequence alignment of the three vertebrate CRYs: mCRY1, zCRY1a, and zCRY3, and E. coli CPD photolysate (EcCPD). Amino acid residues of zCRY1a and -3 and E. coli CPD photolysate that are identical with those of mCRY1 are indicated by dashes. Missing amino acid residues are indicated by the asterisk (*) (see text). Regions exchanged in the chimeras are boxed in white, green, yellow, light blue, red, or dark blue. B, 126–196, 197–263, and 264–293, respectively, are designated RD-2a, RD-1, and RD-2b (see text). Repressor type-specific amino acids in RD-2a and RD-1, which are well conserved in all repressor-type CRYs but differ in the non-repressor-type CRY (zCRY3), are shown by underlining in the mCRY1 sequence. B, structural model of zCRY1a constructed by use of the crystal structure of E. coli photolysate. Although zCRY1a has a limited degree of conservation with E. coli CPD photolysate (25% identical and 41% similar) as seen in A, the model structure obtained has quality corresponding to a crystal structure with 2.0 to 2.5-A resolution (see “Experimental Procedures”). The model does not contain the C-terminal 168 residues (431–598) of zCRY1a because the C-terminal region of zCRY1a and E. coli photolysate show no homology. The six regions used to construct the chimeras are showing in color: 1–155 (white), 126–196 (green), 197–263 (yellow), 264–293 (light blue), 294–419 (red), and 420–431 (dark blue). FAD cofactor is shown in the ball-and-stick models. C, scheme representation of zCRY1a and the regions exchanged in the chimeras.
2A, 19 chimeras composed of reciprocal domain swaps were generated between zCRY1a and -3.

**Determination of Those Sequence Elements of zCRY1a Sufficient for Transcriptional Repression**—Effects of the chimeric zCRYs on zCLOCK:zBMAL-mediated transcription were examined in a luciferase reporter gene assay. As reported elsewhere (16, 20), co-expression of zCRY1a efficiently inhibits zCLOCK1:zBMAL3-mediated transcription (Fig. 2B, lane 3), whereas that of zCRY3 does not (lane 4). First, two chimera series (chimeras 1–8), in which the N- or C-terminal regions of zCRY1a were replaced sequentially by the corresponding regions of zCRY3, were tested. Two chimeras, 3 with amino acids 197–557 and 6 with 1–293 of zCRY1a, maintained transcriptional repression activity (lanes 7 and 10), whereas their reciprocals, chimeras 4 and 5, lacked that activity (lanes 8 and 9). This showed that the region between amino acids 197 and 293 of zCRY1a is necessary for transcriptional repression. In fact, chimera 9, which had amino acids 197–293 of zCRY1a, had repressor activity (lane 13), whereas its reciprocal, chimera 10, did not (lane 14). To identify precisely the critical domain, the 197–293 region was divided into two subregions, 197–263 and 264–293. Four chimeras, 11, 12, 13, and 14, carrying each region, were tested for repressor activity. Chimera 11 with the 197–263 residues of zCRY1a had activity (lane 15), whereas chimera 13 with the rest of the region (264–293) lacked it (lane 17), evidence that the 197–263 region of zCRY1a is sufficient to repress zCLOCK1:zBMAL3-mediated transcription. Consistent with this conclusion, chimera 14, with all zCRY1a sequences except the 264–293 region, which was replaced by zCRY3, had repressor activity (lane 18). Unexpectedly, chimera 12, in which the 197–263 region of zCRY1a was replaced with that of zCRY3, also had repressor activity (lane 16). This suggests that besides the 197–263 region of zCRY1 sufficient for repressor activity, a second region also has activity. This second region must combine the separate regions because the two regions of chimeras (chimeras 1–8), in which the N- or C-terminal regions of zCRY1a were replaced sequentially by the equivalent regions of zCRY3, showed no evidence of the presence of a second region. Furthermore, one of the separate regions must be the 264–293 region of zCRY1a because its presence in chimera 12 is the only difference between chimeras 10 and 12; the latter having lost activity, the former retaining it. We therefore generated three additional chimeras, in which the 264–293 region of zCRY1a was combined with one of the remaining zCRY1a regions: 1–125 (chimera 15), 126–196 (chimera 16), both 1–125 and 294–557 (chimera 17). Of these three chimeras, 16 alone had repressor activity. Chimera 18, with only the 126–196 region of zCRY1a, had no activity (lane 22). The second region that confers repressor activity on zCRY3 therefore is a combination of the 126–196 and 264–293 regions.

The expression levels of the chimeras that lacked transcriptional repressor activity were examined. After transfection into NIH3T3 cells of the expression vectors that encoded each chimera, the cell lysates were analyzed by Western blotting. As shown in Fig. 2C, all the chimeras lacking repressor activity were expressed at a level comparable with that of zCRY1a, or chimera 9, a potent transcriptional repressor. This excludes the possibility that attenuation of transcriptional repressor activity in the chimeras was because of protein instability.

Two regions in zCRY1a that are responsible for the repression of CLOCK:BMAL-mediated transcription were identified: amino acids 197–263 of zCRY1a, and a combination of residues 126–196 and 264–293 of zCRY1a. Each of these regions is sufficient for repressor activity because each alone has independent activity. For simplicity, the respective regions 197–293, 126–196, and 264–293 hereafter are designated RD-1,
RD-2a, and RD-2b (Fig. 1, A and C).

Determination of the Sequence Elements of zCRY1a Required for Its Interaction with zCLOCK1 or zBMAL3—For the repression of CLOCK:BMAL-mediated transcription, CRY association with CLOCK and BMAL is important (8, 19, 36). Recently, we showed that zCRY1a associates with both the zCLOCK1 and zBMAL3 proteins, whereas zCRY3 does not (20). We therefore used chimeras to examine whether the three regions RD-1, RD-2a, and RD-2b are sites of interaction with CLOCK and BMAL. The ability of each chimera to interact with zCLOCK1 and zBMAL3 was tested (Fig. 3A).

First a mammalian two-hybrid assay was used (Fig. 3B), in which zCRY fused to the GAL4 DNA-binding domain (GAL4) was co-expressed with zCLOCK1 or zBMAL3 fused to the VP16 transactivation domain (VP16) in NIH3T3 cells. If GAL4-zCRY interacts functionally with VP16-fused protein, VP16 would be recruited to the vicinity of the promoter and cause transactivation. Four chimeras, 11, 13, 16, and 18, which bear the RD-1 or RD-2 regions, were tested. When co-expressed with VP16-zCLOCK1 or VP16-zBMAL3, chimera 11 with the 196–263-(RD-1) region of zCRY1a caused transactivation (Fig. 3B, lanes 4 and 15), indicative that the 196–263-(RD-1) region of zCRY1a is sufficient for association with the CLOCK:BMAL heterodimer. Chimera 16 with both RD-2a and RD-2b also caused transactivation (lanes 8 and 19), whereas chimeras 13 and 18 with RD-2a or RD-2b did not (lanes 6, 9, 17, and 20). The presence of both RD-2a and RD-2b in CRY therefore is necessary for interaction with CLOCK and BMAL, as well as for transcriptional repression activity.

In the nucleus, CLOCK and BMAL form a heterodimer that functions as a transcriptional activator, therefore CRY must enter the nucleus to interact with the heterodimer and subsequently repress its activity (7, 36). One reason why two distinct regions are needed for transcriptional repression could be that one is responsible for the nuclear localization of CRY and the other for direct interaction with the heterodimer. To examine this probability, interactions also were investigated by immunoprecipitation analysis (Fig. 3C). To exclude the possibility that interaction is hard to detect because of different subcellular localizations of the proteins, two kinds of cell lysates that expressed different proteins were mixed in vitro and incubated to form a complex, after which immunoprecipitation assays were done. Two types of COS7 cell extracts were prepared; one derived from COS7 cells transfected with plasmids encoding FLAG-zCRY1a and zBMAL3-V5, the other from cells transfected with VP16-zCRY1a, zCRY3, or a chimera. These extracts first were mixed, incubated on ice, then underwent immunoprecipitation by the anti-FLAG antibody. The proteins precipitated were examined by Western blotting. Chimera 18 with the RD-2a region of zCRY1a and chimera 11 with the RD-1 region co-immunoprecipitated with zCLOCK1-zBMAL3 (Fig. 3C, lanes 2 and 3), whereas chimera 17 with the RD-2b residues of zCRY1a, did not (Fig. 3C, lane 5). These immunoprecipitation results indicate that the RD-2a and RD-1 regions of zCRY1a are the sites of interaction with the zCLOCK1-zBMAL3 heterodimer, and that either region is sufficient for that interaction. They also suggest that the RD-2b region, an element in the second region, must have some other function. For the two-hybrid assay, the interacting proteins must be present together in the nucleus to transactivate the reporter gene. For the immunoprecipitation assay used in this study, however, the proteins do not need to be co-localized. The results presented in Fig. 3C therefore indicate that the sequence elements responsible for nuclear localization are present in the RD-1 and RD-2b regions of zCRY1a. Whether these regions have the ability to localize the zCRY protein in the nucleus therefore was examined.

Determination of the zCRY1a Regions That Regulate Subcellular Distribution—Elsewhere, we reported that zCRY1a is located in the nucleus, whereas zCRY3 mainly is distributed in the cytoplasm (20). The chimeras therefore also can be used to map the regions responsible for subcellular localization. VP16-tagged or non-tagged chimeric proteins were expressed in NIH3T3 cells, and their cellular locations were determined by immunofluorescence (Fig. 4). Subcellular locations were the cytoplasm (N < C), both the cytoplasm and nucleus (n = C), or the nucleus (N > C).

Four chimeras, 11, 13, 16, and 18, which bear one or two of the RD-1, RD-2a, and/or RD-2b regions, were tested for cellular
localization (Fig. 4, lanes 7–18). Except for chimera 18, they were mainly in the nucleus. This is consistent with our prediction that the presence of either the RD-1 or RD-2b regions of zCRY1a are sufficient for the nuclear localization of zCRY and that the RD-2a region lacks such activity.

Identification of a Functional NLS within the Repressor-type of CRY—Proteins larger than 48 kDa require a specific sequence, the nuclear localizing signal (NLS), to be targeted to the nucleus (37). The RD-1 and RD-2b regions are responsible for the nuclear localization of zCRYs. Our search for the consensus NLS sequence in zCRY1a found an NLS-like sequence (residues 265–282) in the RD-2b region. This sequence is highly conserved in repressor-type CRYs from different species but varies substantially in zCRY3 (Fig. 5A). pGFP-zCRY-NLS constructs were generated to determine whether the sequence functions in the nuclear import of zCRY. Amino acids 265–282 of zCRY1a or zCRY3 were inserted into the C-terminal end of GFP, which, respectively, generated pGFP-zCRY1a-(265–282) or pGFP-zCRY3-(265–282). Their subcellular localizations then were determined after transfection into NIH3T3 cells (Fig. 5B). GFP-zCRY1a-(265–282) mainly was limited to the nucleus, whereas the control GFP was present in both the cytoplasm and nucleus (Fig. 5, B and C, lanes 1–6). Unlike GFP-zCRY1a-(265–282), GFP-zCRY3-(265–282) was mainly distributed in both the cytoplasm and nucleus (Fig. 5, B and C, lanes 7–9). These findings indicate that residues 265–282 of zCRY1a constitute a functional NLS and that the distinct subcellular localizations in zCRY1a and -3 depend in part on different NLS activities.

In the RD-1 region, there was no consensus NLS sequence. The GFP-RD-1 fusion protein (pGFP-zCRY1a-(197–263)) was mainly restricted to the nucleus (data not shown). The RD-1 region therefore carries an unknown NLS.

The NLS-(265–282) identified in zCRY1a is well conserved among the repressor-type CRYs of various organisms (Fig. 5A). To determine whether the identified sequences also function as an NLS in other repressor-type CRYs, we determined whether disruption of the NLS of mouse CRY (mCRY) affects its subcellular distribution. Two types of NLS-mutated mCRY1s (mut-2 and mut-9) were generated by replacing two or nine amino acids of the NLS with the corresponding amino acids of zCRY3 (Fig. 6A). Both mutant proteins were expressed at levels comparable with the level of wild-type mCRY1 (Fig. 6B), indicative that the two types of substitutions did not affect the stability of the mCRY1 protein.

The subcellular distribution of each mCRY1 mutant was investigated (Fig. 6, C and D). When expressed in NIH3T3 cells, wild-type mCRY1 mainly was detected in the nucleus.
Interestingly, both types of mutations in the NLS (amino acids 265–282) of mCRY1 produced cytoplasmic distribution of mCRY1 (Fig. 6, C and D, lanes 4–9), evidence that the intact NLS (residues 265–282) of mCRY1 is necessary for its nuclear localization.

**DISCUSSION**

As the focus of this study was the identification of the functional regions of repressor-type CRY, initially deletion analysis was carried out. It was unsuccessful, however, because any type of deletion in the N-terminal-conserved regions of the CRYs (mCRYs and zCRYs) resulted in gross functional defects in the proteins (data not shown). The results indicated that the overall conformation of the N-terminal region is required for the CRY function, as previously reported for the dCRY protein (38). To circumvent the deletion analysis problem, zCRYs were used. Two types of zCRY proteins are present in the zebrafish. One represses CLOCK:BMAL-induced transcription (repressor-type, zCRY1a, -1b, -2a, and -2b), the other does not (non-repressor-type, zCRY3 and -4). Of these CRYs, repressor-type zCRY1a and non-repressor-type zCRY3 share a high degree of sequence homology, but differ in several characteristics, including interaction with the CLOCK:BMAL1 heterodimer and subcellular localization (16, 19, 20). This sequence homology allowed the switch of a region of the repressor-type CRY1a with the corresponding region of the non-repressor-type CRY3 without altering the wild-type conformation (Fig. 1). The resulting zCRY1a-3 chimeras were used to determine which CRY regions are required for transcriptional repression, interaction with other clock proteins, and regulation of the subcellular distribution. Either RD-2a (residues 126–196) or RD-1 (residues 265–282) are required for interaction with other clock proteins, and either

**Fig. 5. Identification of the NLS in repressor-type CRY.** A, comparison of the putative NLSs of zCRY1a and other repressor-type CRY proteins. Amino acid residues identical to those of zCRY1a are shown in white letters on a black background. Numbering starts from the N terminus of each protein. In zCRY2a numbers are omitted because its full-length sequence has yet to be reported. zCRY, xCRY, cCRY, mCRY, and hCRY, respectively, represent zebrafish CRY, Xenopus CRY, chicken CRY, mouse CRY, and human CRY. B, representative examples detected by GFP fluorescence of the subcellular localization patterns of GFP, GFP-zCRY1a (amino acids 265–282), and GFP-zCRY3 (amino acids 265–282). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). C, quantitative analysis of the subcellular localizations of GFP, GFP-zCRY1a (amino acids 265–282), and GFP-zCRY3 (amino acids 265–282), as shown in Fig. 3C.
RD-1 or RD-2b (residues 264–293) are required for nuclear localization of CRY. Both of these functions are prerequisites for transcriptional repressor activity. Thus far, crystal structures have been determined for four members of the DNA photolyase/Cryptochrome family; three CPD photolyases, one each from *E. coli*, *Anacystis nidurans*, and *Thermus thermophilus* (39–41), and one Cryptochrome from *Synechocystis* sp. (42). These proteins have similar three-dimensional structures. They are folded in two domains, an α/β and a helical domain connected by a long interdomain loop. The helical domain has all the residues that bind FAD. FAD binds noncovalently at the bottom of the cavity formed between the two distinct lobes of that domain. As stated in the description of the strategy for determining the domain to be exchanged, the RD-2a region constitutes the major part of the interdomain loop, RD-1 and RD-2b covering the N-terminal third of the helical domain (Figs. 1C and 7). The amino acid residues that are well conserved in all the repressor-type CRYs, but not in non-repressor-type CRY (zCRY3), are the most promising candidates for the critical site of each function. These residues are Ser-129, Leu-135, Gly-143, Tyr-150, Ser-158, and Met-160 in RD-2a, and Leu-205, Ala-208, Met-239, Asn-240, and Asn-242 in the RD-1 region. Residues in the NLS of RD-2b are dark blue. The cavity is indicated by an arrow. Two views are presented to show clearly the position of the repressor-type-specific amino acid residues.

CRY is thought to act as a transcriptional repressor by interacting directly with the CLOCK:BMAL heterodimer in the nucleus (7, 8, 19, 38); and both interactions with the heterodimer and nuclear translocation seem to be essential for the transcriptional repressor activity of CRY. Our findings provide several lines of evidence that support this. First, the area responsible for nuclear localization and for interaction with CLOCK and BMAL1 are separable into two distinct regions: the former is RD-2b, the latter is RD-2a. Transcriptional repressor activity occurred only when both regions were present in the molecule. Second, there was good correlation between the transcriptional repressor activity and nuclear localization ability in several types of chimeric or mutated CRYs. We identified a functional CRY NLS (residues 265–282) in zCRY1a and mCRY1 that is highly conserved in repressor-type CRYs of different organisms. Mutations in the NLS of mCRY shift subcellular localization from the nucleus to the cytoplasm and decrease its ability to repress CLOCK:BMAL-induced transcription (data not shown). Taken together, these findings clearly indicate that both nuclear localization and interaction with the CLOCK:BMAL1 heterodimer are prerequisites for the transcriptional repressor activity of CRY.

Initially, we speculated that mCRY1 and zCRY1a have identical structures and functions. This was based on two facts: the high homology of the primary structures of both CRYs and the *in vitro* abilities of these CRYs to repress the transcription activated by mouse- or zebrafish-derived CLOCK and BMAL1 (16, 20). The present findings are consistent with this speculation. Except for one amino acid in each region (D203E and K274R) (Fig. 1A), the amino acid sequences in the RD-1 and RD-2b regions are identical. Furthermore, the RD-2b region functions as a nuclear localizing domain in mCRY1 (Fig. 6). The amino acid sequences of the RD-2a region vary to some extent, but the sequences of the N-terminal halves of RD-2a, in which the repressor type-specific amino acid residues (Ser-129, Leu-135, Gly-143, Tyr-150, Ser-158, and Met-160) are clustered, are well conserved in the two CRYs (Fig. 1A). These observations confirm our speculation. We also tried to determine the interaction site at the amino acid level in RD-1 and RD-2a, but were unsuccessful because no point mutation at any of the repressor type-specific amino acid residues had a clear effect on the repressor activity of chimeric zCRYs (data not shown). Not just one, but several amino acid residues are responsible for that activity. The introduction of clustered mutations at these residues will help us to determine which are the critical amino acids in these regions and providing a clearer understanding of the regulatory mechanism of this fascinating CRY protein.

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Functional and Structural Analyses of Cryptochrome: VERTEBRATE CRY REGIONS RESPONSIBLE FOR INTERACTION WITH THE CLOCK:BMAL1 HETERODIMER AND ITS NUCLEAR LOCALIZATION

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