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A Mammalian Origin of Bidirectional DNA Replication within the Chinese Hamster *RPS14* Locus†

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Two complementary experimental approaches have been used to identify a chromosomal origin of bidirectional DNA replication within or immediately downstream of the Chinese hamster ribosomal protein S14 gene (*RPS14*). The replication origin, designated ori_{S14}, maps within a 1.6- to 2.0-kbp region of *RPS14* that includes the gene's third and fourth introns, exons IV plus V, and ~500 bp of proximal downstream flanking DNA. The nucleic acid sequence encoding ori_{S14} closely resembles the other mammalian chromosomal replication origins whose primary structures are known. It contains DNA binding sites for a large number of transcription factors, replication proteins, and mammalian oncogenes as well as several dinucleotide repeat motifs, an AT-rich region, and a sequence that is likely to bend the DNA. In contrast to the other well-characterized mammalian replication origins, which are autosomal and therefore carried as two copies per somatic cell, ori_{S14} is encoded by single-copy DNA within a hemizygous segment of chromosome 2q in CHO-K1 cells. Also, other known mammalian replication origins are situated in nontranscribed, intergenic DNA, whereas the DNA sequence encoding ori_{S14} substantially overlaps the transcribed portion of a constitutively expressed housekeeping gene.

Chromosomal DNA synthesis in higher eukaryotes is initiated from multiple, independent, bidirectional origins of replication (OBRs) (25). A pair of replicating DNA forks diverge from each origin to synthesize daughter duplex DNAs via a semidiscontinuous mechanism (12, 14, 15). Individual OBRs initiate DNA synthesis at specific times during S phase (3, 9, 19, 32, 45). Indeed, temporal programming of OBRs appears to be an important, if not essential, step in the establishment of alternative developmental pathways in metazoan organisms. For example, the short mitotic cycles observed in early-cleavage-stage embryos reflect the activities of closely spaced OBRs, many of which are inactive in differentiated adult cells (12). Similarly, transcriptional activation of diverse, developmentally regulated genes often involves the reprogramming of their replication to early times in S phase (2, 11, 13, 16, 17, 23, 27, 32, 46, 53).

To investigate molecular mechanisms which mediate the activities of metazoan replication origins and account for their dynamic regulation, it is necessary to isolate and characterize them as cloned DNA sequences. Although numerous replication origins have been isolated from bacterial, yeast, and viral chromosomes, only a few have been obtained from mammalian chromosomes. In large part this is due to the fact that prokaryotic, viral, and yeast origins support autonomous replication of episomal DNAs, whereas mammalian origins usually do not (14). As an alternative, several sensitive assays for chromosomal origin function based on physical and chemical properties of replicating duplex DNAs have been developed (50) and used to identify OBRs which reside near human c-*myc* (28, 48) and *aprt* (22), the murine adenosine deaminase locus (10, 51), and the Chinese hamster *dhfr* (dihydrofolic acid reductase) (1, 5, 30) and rhodopsin (20) genes.

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As described in the accompanying report (44), during a recent investigation of the DNA methylation patterns within the Chinese hamster ovary (CHO) cell gene encoding ribosomal protein S14 (*RPS14*) (43), we observed a densely methylated DNA sequence whose presence was strictly correlated with the cells' replication state. Our observations suggested that the methylated nucleic acid sequence might be important for chromosomal DNA replication and prompted us to examine the CHO *RPS14* gene for an OBR. Accordingly, we have used two independent assays for replication origin activity to identify a chromosomal OBR within the 1.6 to 2 kbp of DNA between *RPS14*'s third intron and a site ~550 bp downstream of its last exon. This chromosomal replication origin is designated ori_{S14}.

MATERIALS AND METHODS

Materials. Tissue culture media and fetal bovine serum were purchased from GIBCO BRL Life Technologies, Inc. A purified murine monoclonal antibody directed against bromodeoxyuridine (BrdU) was provided by Becton Dickinson Immunocytometry Systems, Inc. Amino acids, aphidicolin, rabbit anti-mouse immunoglobulin G, and 5-bromo-2-dUTP (BrdUTP) were obtained from Sigma Chemical Co. T7 and SP6 RNA polymerases as well as *Taq* DNA polymerase were products of Promega Corp. GeneScreen Plus filter membranes, [α-^{32}P]dATP, and [α-^{32}P]dCTP (both 3,000 Ci/mmol) were purchased from DuPont NEN Research Products. [2,8-^{3}H]dA (15 Ci/mmol) was a product of Moravek Biochemicals. All other enzymes and reagents were obtained from commercial suppliers indicated previously (4, 31, 35, 43).

DNA sequences. The Chinese hamster *RPS14* gene (5,300 bp) is listed in GenBank under the locus name CRURPS14A (accession no. M35008). A complete genomic DNA clone of this sequence, pGS14-45, has been described elsewhere (39). Oligodeoxynucleotide primers necessary to amplify unique segments of *RPS14* by PCR (33) were synthesized by the Biotechnology Core Facility at Kansas State University (Table 1). A plasmid clone of Chinese hamster cell *ori*-β (pX24),

TABLE 1. Synthetic oligonucleotides used in this study

Oligonucleotide	Location (5′→3′)[a]	Sequence (5′→3′)[b]
P1	2334→2359	5′-CTCAATTATCCTCAGCTACACGCACAT-3′
P2	2568→2545	5′-ACAAAAGCAGATCCATGCCTTCATT-3′
P3	3160→3186	5′-TTGAGCTGAACAGTGATGCTTAAATGA-3′
P4	3390→3364	5′-ATCTTTCCACTCAGTAGCCACCTATTC-3′
P5	5080→5106	5′-ACGAATTCTTGCAGGCTGGCTTTCTGAG-3′
P6	5300→5273	5′-GAGGATCCCAAGTAGACCATATTAAACT-3′
P7	~7900→7926[c]	5′-AGCCAGCTGTCCTTTATATGCTTGGCA-3′
P8	~8400→8371[c]	5′-TACTCCTTACATTGTCATATTCATGAATG-3′

[a] Locations within the CHO *RPS14* sequence are specified according to the index used in GenBank entry M35008.
[b] Nucleic acid sequences are derived from the cloned CHO *RPS14* gene pGS14-45 (39).
[c] Determined from a 0.5-kbp *Bgl*II-*Bam*HI fragment which is located ~3 kbp downstream of *RPS14* exon V (39).

located downstream of the *dhfr* locus on chromosome 2p, was generously provided by Melvin DePamphilis and David Gilbert (Roche Institute of Molecular Biology).

Cell culture. CHO-K1 cells (ATCC CCL61) were propagated as monolayer cultures at 37°C in antibiotic-free Dulbecco's modified Eagle's medium plus 10% (vol/vol) fetal bovine serum supplemented with 10^{-4} M glycine and proline (DMEM-PG). Cells synchronized to the G_1-S phase boundary were obtained by treatment with isoleucine-free medium for 50 h followed by reversal into DMEM-PG containing 5 μg of aphidicolin per ml for 12 h, using a protocol described by others (6, 24).

Identification of a chromosomal origin by analysis of replication fork asymmetry. The rationale for the approach used is based on the observation that replication of duplex DNA is a semidiscontinuous process (26) and chromosomal replication forks are therefore asymmetric structures (40). As replication forks move through duplex DNA, DNA on the leading side of the fork is synthesized processively while DNA on the retrograde side is replicated through discontinuous, short DNA intermediates, i.e., Okazaki fragments (12). As a consequence, Okazaki fragments are complementary to only one of the two template strands within each replication fork. Therefore, as two replication forks diverge from an OBR, one expects a switch in nascent Okazaki fragment template strand specificity. This provides an empirical criterion by which to recognize and map chromosomal replication origins in higher eukaryotes (6).

Accordingly, analysis of nascent Okazaki fragment strand specificity within the CHO cell *RPS14* locus was carried out as described by Burhans et al. (6). Briefly, Okazaki fragments synthesized during early S phase and labeled with both BrdU and [^{32}P]dATP were purified and used as filter hybridization probes against unique, single-stranded *RPS14* RNA target sequences. To isolate radiolabeled early-S-phase Okazaki fragments, CHO cells were presynchronized to the G_1-S phase boundary (see above) and allowed to initiate S phase for 5 min. The cells were permeabilized by treatment with Nonidet P-40, and replicating DNAs were labeled in vitro with BrdUTP and [^{32}P]dATP for 1.5 min at 34°C. Labeling reactions were terminated, and the samples deproteinized by treatment with sodium dodecyl sulfate (SDS) and proteinase K (90 min at 37°C). Chromosomal DNAs were recovered by ethanol precipitation, denatured in boiling water, and size fractionated by alkaline agarose gel electrophoresis. Radioactive DNAs in the size range of 50 to 250 nucleotides (Okazaki fragments) were electroeluted and treated with 0.2 N NaOH for 24 h at 37°C to hydrolyze contaminating RNA. After neutralization, the DNAs were incubated with a mouse anti-BrdU monoclonal antibody and then with rabbit anti-mouse immunoglobulin G to immunoprecipitate nascent, BrdU-labeled, single-stranded Okazaki fragments. The immunoprecipitates were collected by centrifugation, redissolved in Tris-EDTA buffer, and used as hybridization probes against dot blot filters containing single-stranded RNAs encoding unique portions of the *RPS14* locus.

Five DNA fragments derived from a cloned copy of the CHO *RPS14* gene, pGS14-45 (39), were determined to contain only unique nucleic acid sequences by using them as probes to analyze Southern blots of CHO genomic DNA digested with *Hin*dIII (not shown) and *Pst*I (Fig. 1). DNA probes which visualized single bands on these blots were judged to consist predominantly, if not exclusively, of single-copy sequences and were subcloned into the bacterial plasmid pGEM-1 (Promega

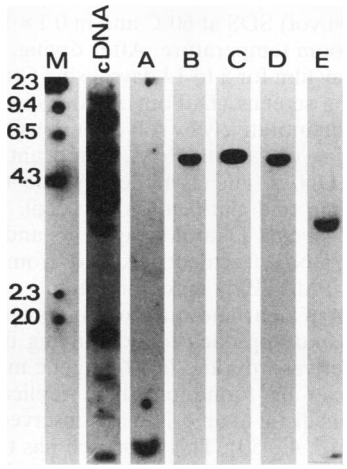

FIG. 1. CHO *RPS14* marker fragments A to E are composed of unique, single-copy genomic DNA sequences. CHO genomic DNA was digested with *Pst*I, and the resulting fragments were resolved by electrophoresis on a 0.8% agarose gel (34). Each lane of the gel contained 10 μg of the DNA fragments, which were blotted to a GeneScreen Plus filter membrane. After the membrane was sliced into six strips, each strip was analyzed individually by hybridization with a different nick translated [^{32}P]DNA probe. The entire complement of CHO DNA fragments with sequences complementary to the S14-coding sequence was detected by using a full-length S14 cDNA, pCS14-11 (37), as the probe (lane cDNA). Lanes A to E were analyzed by using the ^{32}P-labeled marker DNA fragments A to E (see text and Fig. 2), respectively, as hybridization probes. The positions of the molecular weight markers (λ *Hin*dIII fragments) were recorded by using radioactive ink and are illustrated in lane M. The size (indicated in kilobases) of each single band detected in lanes A to E is consistent with the known restriction map of the CHO *RPS14* locus (39).

Corp.) for cell-free transcription using T7 and SP6 RNA polymerases (4). Fragment orientations within the plasmid's polylinker were determined by DNA sequence analysis (42) using T7 and SP6 promoter oligonucleotides (Promega) as primers. In this way, pGEM-1 clones of DNA fragments A to E (Fig. 1 and 2) were prepared from *RPS14* introns 1, 2, and 3 and from the proximal downstream flanking sequence. Fragment A is a *Pst*I restriction fragment encoding *RPS14* nucleotides 1085 to 1603, fragment B is a PCR-amplified sequence (*RPS14* nucleotides 2130 to 2570) that has been described previously as pGS14-41 (39), fragment C is an *Acc*I-*Afl*III restriction fragment encoding nucleotides 3074 to 3510, fragment D is a *Bgl*I-*Eco*RI fragment containing nucleotides 5140 to 5300, and fragment E is a 500-bp *Bgl*II-*Bam*HI fragment located ~3 kbp downstream of exon V (39). These DNA fragments were transcribed into single-stranded RNA targets by using T7 and SP6 RNA polymerases, freed of DNA template by digestion with DNase I, and purified as described previously (4). Target RNAs were dot blotted to GeneScreen Plus membranes for hybridization with the [^{32}P]dATP-labeled Okazaki fragments.

RNA targets (10 μg) were dissolved in 50% deionized formamide–6% formaldehyde and incubated at 50°C for 60 min before application to GeneScreen Plus filter membranes by using a dot blot manifold. The membranes were air dried, baked at 80°C for 2 h, and treated with a prehybridization solution containing Ficoll, polyvinylpyrrolidone, and sodium pyrophosphate (0.2% [wt/vol] each), 1 M NaCl, and 1% (wt/vol) SDS for 4 h at 60°C. ^{32}P-Okazaki fragments (10^6 cpm) then were added to the prehybridization mix, and incubations were continued for an additional 16 h. Filters were rinsed twice in 2× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]) at room temperature and then washed for 60 min in 2× SSC–1% (wt/vol) SDS at 60°C and in 0.1× SSC–0.1% SDS for 15 min at room temperature. After drying, the filters were exposed to X-ray film for 5 to 14 days, using Cronex Lightning-Plus intensifying screens (DuPont). Autoradiographic signals were analyzed quantitatively by soft laser densitometry. To test for equal loading of target RNAs, significant contamination with template DNAs, and asymmetries due to the Okazaki fragment labeling and purification protocol, dot blots were stripped of ^{32}P-labeled Okazaki fragments and reprobed with duplex *RPS14* DNA fragments excised from pGEM-1 and labeled with [^{32}P]dATP by nick translation.

Identification of a chromosomal origin by nascent DNA size analysis. A second approach for identifying unique chromosomal OBRs derives from the fact that gene markers adjacent to an origin can be found on short replication products, whereas markers distal to an origin are observed only in longer nascent DNAs (6, 47, 50). This approach has the merit of not requiring synchronized cell populations and therefore avoids artifacts which potentially can result from the synchronization protocol. To implement this approach, we followed the PCR-based strategy described by Vassilev et al. (49) as modified by Virta-Pearlman et al. (51).

Exponentially growing monolayer cultures of CHO cells were labeled with [^{3}H]dA (3 μCi/ml) and 0.02 mM BrdU (Boehringer Mannheim) for 15 min at 37°C. After removal of the labeling medium, cells were lysed in a solution containing 50 mM Tris-HCl (pH 8.0), 1 M NaCl, 10 mM EDTA, and 0.5% (wt/vol) SDS. Lysates were deproteinized by treatment with proteinase K (10 μg/ml) for 14 h at 37°C followed by phenol-chloroform extractions and ethanol precipitation. Resulting DNAs were size fractionated by electrophoresis through 1% (wt/vol) alkaline agarose gels for 18 h at 2 V/cm together with DNA size standards (bacteriophage lambda *Hin*dIII DNA fragments and a 1-kbp DNA ladder purchased from GIBCO BRL). As illustrated in Fig. 4, gels were sliced into discrete molecular-size intervals based on these markers, and chromosomal DNAs within each slice were recovered by electroelution, phenol extraction, and ethanol precipitation. [^{3}H]dA-BrdU-labeled DNAs within each fraction were immunoprecipitated as described above.

Equimolar amounts of each nascent BrdU-DNA preparation (based on ^{3}H radioactivity) were assayed for the *RPS14* sequence-tagged sites (STSs) illustrated in Fig. 4 by PCR using the primers listed in Table 1. PCR amplification mixtures (50 μl) contained 30 pmol of size-fractionated BrdU-DNA template, 360 pmol of each oligonucleotide primer, 2.5 U of *Taq* polymerase, and all four deoxynucleoside triphosphates (0.2 mM each) in 10 mM Tris-HCl (pH 9.0)–50 mM KCl–0.1% (vol/vol) Triton X-100. STS markers were amplified by 30 cycles of a protocol consisting of 1 min of denaturation at 94°C, 1 min of annealing at 55°C (or 50°C for marker E), and 2 min of elongation at 72°C. STS markers amplified from each fraction of nascent DNA were loaded on to a 1.5% neutral agarose gel formed in 100 mM Tris-borate (pH 8.3)–2 mM EDTA–0.5 μg of ethidium bromide per ml and electrophoresed for 2 h at 10 V/cm. The *RPS14* OBR was mapped relative to the STS markers by determining the shortest nascent DNA template fraction that contains each STS (51). This approach avoids the necessity to quantitate STS markers within each amplified fraction precisely (49) but renders each measurement a maximal estimate of the distance between the STS its associated replication origin.

RESULTS

Analysis of Okazaki fragment DNA replication intermediates within the CHO cell *RPS14* locus. As described above, the assay was designed to detect a transition in the distribution of DNA biosynthetic intermediates that derive from the retrograde side of chromosomal replication forks traversing the *RPS14* locus (14).

Monolayer cultures of CHO cells synchronized to early S phase were harvested, and their nuclei were purified. Nascent, single-stranded replication intermediates initiated in vivo were labeled with BrdUTP and [α-^{32}P]dATP in vitro as described in Materials and Methods. ^{32}P-Okazaki fragments then were purified by electrophoresis and immunoprecipitation. In a preliminary series of experiments designed to test the efficacy of our methods, we used the ^{32}P-Okazaki fragments to probe dot blots containing single-stranded target RNAs transcribed from cloned DNA fragments of the CHO *dhfr ori*-β region studied by others (6). As reported, our implementation of the method employing single-stranded RNA targets detected a DNA strand switch in Okazaki fragment complementarity in precisely the same location documented by Burhans et al. (6).

To identify Okazaki intermediates which originate from the *RPS14* locus on CHO chromosome 2q, five unique DNA marker sequences distributed across the gene (labeled A to E in Fig. 2) were subcloned into pGEM-1 and transcribed into five pairs of single-stranded RNA target molecules, using SP6 and T7 RNA polymerases. The RNAs were dot blotted to filter membranes and probed with ^{32}P-labeled Okazaki fragments.

In the experiment illustrated by Fig. 2, early-S-phase Okazaki fragments preferentially hybridized to antisense RNA targets (5′→3′, right to left as drawn) at marker sites A, B, and C and to sense RNA targets (5′→3′, left to right as drawn) at marker sites D and E. No Okazaki fragments complementary to sense-strand RNAs were detected at marker sites A and B. Similarly, no Okazaki fragments complementary to antisense

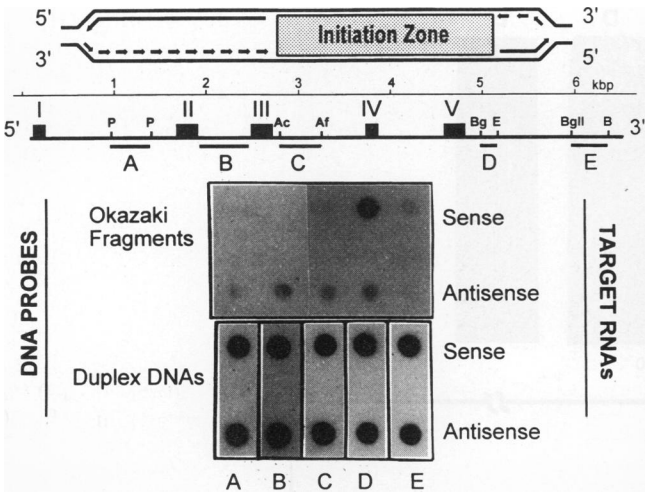

FIG. 2. A transition in Okazaki fragment distribution signals a chromosomal OBR located within or immediately adjacent to the Chinese hamster *RPS14* gene. Equal aliquots (10 μg) of unique strand-specific *RPS14* marker RNAs (A to E) were dot blotted to a GeneScreen Plus filter and probed with 10^6 cpm of ^{32}P-labeled early-S-phase Okazaki DNA fragments (top autoradiogram). After removal of hybridized Okazaki fragments, the filter was cut into strips containing each marker RNA pair and reprobed with ~10^7 cpm of double-stranded [^{32}P]DNA prepared by nick translation from the cloned marker DNAs (bottom row of autoradiograms). Relative intensities of each dot visualized in the autoradiograms were determined by soft laser densitometry. The distribution of Okazaki fragments within divergent replication forks that traverse *RPS14* in vivo was inferred from the data and is illustrated in the diagram at the top (discontinuous arrows). Restriction endonuclease cleavage sites indicated: P, *Pst*I; Ac, *Acc*I; Af, *Afl*III; Bg, *Bgl*I; E, *Eco*RI; BgII, *Bgl*II; B, *Bam*HI. *RPS14* exons are represented by shaded rectangles labeled I to V. Other details of the analysis are described in Materials and Methods. The molecular scale illustrated is based on the nucleotide numbering system used in GenBank entry M35008.

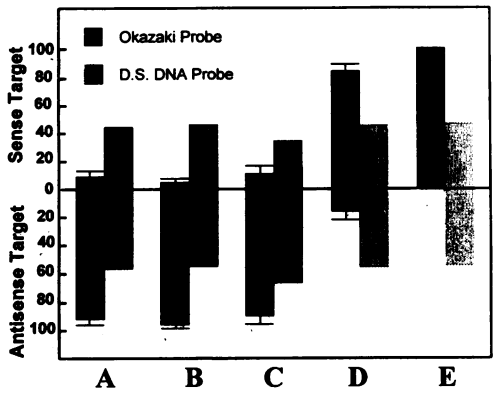

FIG. 3. DNA strand biases displayed by Okazaki fragment probes within the CHO *RPS14* locus. The results of four independent experiments using ^{32}P-labeled Okazaki fragment probes and two experiments using double-stranded (D.S.) DNA probes are summarized. Each analysis was carried out as described in the legend to Fig. 2. Densitometric scans were normalized as the percent intensities recorded in the individual dots of each dot pair, i.e., sense/(sense + antisense) and antisense/(sense + antisense), to accommodate several dots which displayed zero intensity. The normalized values are plotted as means ± standard errors.

RNA were observed at marker E. Densitometric analysis indicated a 12-fold bias toward the antisense target at marker site C and a 5-fold bias toward the sense target at marker D. The transition in Okazaki fragment distribution clearly suggests that an OBR is located within the *RPS14* locus between intron 3 (marker site C) and the proximal downstream flanking sequence (marker site D), i.e., between positions 3510 and 5140.

To verify that equal amounts of the strand-specific RNA target molecules were fixed to the dot blot membranes and that neither the methods used to label and purify Okazaki fragments nor the hybridization protocol was responsible for asymmetries observed, filter membranes were stripped of the Okazaki fragment probes and reanalyzed with double-stranded DNA fragments symmetrically labeled with ^{32}P by nick translation. As also shown in Fig. 2, autoradiographic signals observed in response to double-stranded DNA probes were equally intense on both the sense and antisense target dots at all five *RPS14* marker sites. Ratios of the signals measured against each pair of RNA targets in this experiment displayed a mean of 1.0 ± 0.02.

Densitometric data derived from four additional analyses carried out as described for the experiment shown in Fig. 2 are summarized in Fig. 3. In all four experiments, Okazaki fragment probes produced a much higher signal against the sense-strand target of fragment D (Fig. 2) than they did against the other nine single-stranded targets. In contrast, duplex DNA probes (such as illustrated in Fig. 2) did not preferentially react with the fragment D sense target, nor did they display a significant strand bias. These observations, together with the Southern blots shown in Fig. 1, indicate that the strand biases and marker preferences observed in experiments summarized by Fig. 2 and 3 reflect the DNA composition of Okazaki fragment probes and not the purity or amounts of RNA target molecules used. A similar marker preference was reported previously by Carroll et al. as part of their analysis of a murine replication origin near the adenosine deaminase locus (10). They interpreted the marker preference they observed to indicate that chromosome sequences immediately proximal to mammalian replication origins (i.e., newly initiated replicons) are overrepresented in standard Okazaki fragment preparations. The preference in Okazaki fragment hybridization that we observe toward *RPS14* marker fragment D (Fig. 2) is consistent with this explanation.

Analysis of nascent DNA sizes encoding *RPS14*. To confirm the location of a chromosomal OBR within the CHO *RPS14* gene, we also analyzed the gene by using a second, independent experimental strategy. This approach is based on the expectation that marker DNA sequences that reside near replication origins are contained in newly replicated DNAs of all sizes, whereas marker sequences located distal to replication origins are encoded only by longer nascent DNAs (47, 48, 50).

To carry out this test, exponentially growing CHO cells were pulse-labeled with [^{3}H]dA and BrdU for 15 min at 37°C and then lysed in a detergent-EDTA buffer. Total cell DNAs were extracted and fractionated on the basis of size by alkaline agarose gel electrophoresis. Newly synthesized (i.e., BrdU-containing) single strands of DNA were isolated by immunoprecipitation (see Materials and Methods). Each size fraction of BrdU-DNA then was analyzed for the presence of unique *RPS14* chromosomal sequences by PCR amplification. In the experiment illustrated in Fig. 4, four *RPS14* marker sequences contained within the fragments labeled B to E in Fig. 2 were used.

As explained by Virta-Pearlman et al. (51), the shortest

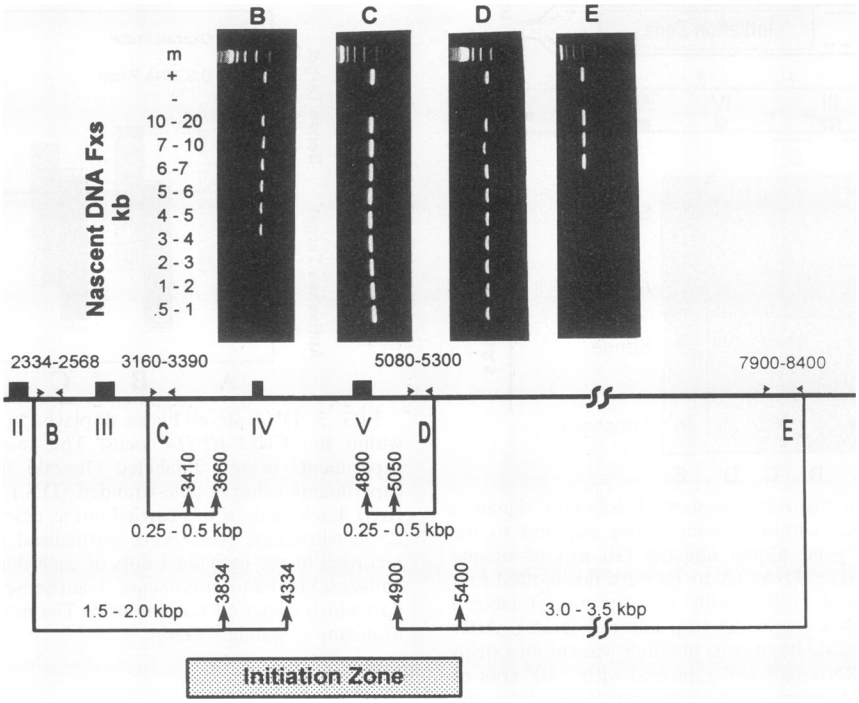

FIG. 4. Analysis of PCR-based STSs confirms the location of a chromosomal OBR within the CHO *RPS14* locus. Single-stranded DNAs replicated by exponentially growing CHO cells in vivo were pulse-labeled with BrdU and size fractionated by alkaline agarose gel electrophoresis (see Materials and Methods). Each size fraction (Fx) of BrdU-DNA was used as a PCR template to analyze four unique STSs within the *RPS14* marker DNA fragments illustrated in Fig. 1 (→ ←). PCR-amplified STS markers were assessed by electrophoresis through neutral ethidium bromide agarose gels (labeled B to E to indicate the primer pair used). The molecular lengths of nascent DNA templates used in each amplification reaction are indicated to the left of the electropherograms. m, lanes that contained DNA size standards; +, lanes loaded with amplification products generated from total CHO genomic DNA templates; −, lanes in which template DNAs were omitted from the amplification reactions. The locations of marker sequences used in this analysis are specified above the restriction map, and the positions inferred for the replication origin (↑) are shown below the map. Chromosomal distances separating each marker and the OBR were estimated from the electrophoretic data (51) and are indicated below the map (see text). Other features of the figure are described in the legend to Fig. 2.

nascent DNA template encoding a marker sequence can be used to estimate the distance between that marker and the chromosomal origin which initiates its replication. If one assumes that forks which diverge from an active OBR traverse surrounding chromosomal DNA at equal rates, then the distance between a unique marker sequence and the OBR from which it was replicated is expected to be one-half the length of the shortest nascent DNA template containing the marker. Accordingly, maps of chromosomal replication origins based on this strategy for PCR analysis of nascent DNA strands are based on three key assumptions: (i) that divergent replication forks migrate through the replicon at constant and equal rates; (ii) that STS markers within nascent (i.e., BrdUrd-containing) DNAs can be detected qualitatively by using unique amplification primer oligonucleotides; and (iii) that nascent, single-stranded BrdUrd-DNAs less than 20 kbp in length can be isolated without significant degradation.

As illustrated in Fig. 4B, a 235-bp STS marker derived from *RPS14* intron 2 (positions 2334 to 2568) was amplified by using primers P1 and P2 (Table 1) from nascent DNA templates whose lengths are equal to or greater than 3 to 4 kbp. Shorter nascent DNAs did not contain the marker. Therefore, as described above, this STS appears to be located within 1.5 to 2 kbp of the active OBR. Similarly, the intron 3 STS (Fig. 2C, positions 3160 to 3390) and the proximal downstream STS (Fig. 2D, positions 5080 to 5300) both resided within 0.25 to 0.5 kbp of the replication origin, since nascent DNAs of all sizes resolved by electrophoresis (0.5 to 20 kbp) encoded them. Finally, the distal downstream STS, defined by primers P7 and P8 (Table 1), was amplified only in response to nascent DNA templates larger than 6 to 7 kbp (Fig. 2E). This positioned STS E 3 to 3.5 kbp away from the origin responsible for its replication.

Because Okazaki fragment probes displayed a reproducible preference for marker sequence D (Fig. 2), which might suggest that marker D includes a repetitive DNA sequence despite the Southern blot patterns observed (Fig. 1), we subcloned 10 independent DNA fragments amplified from CHO DNA by using primers P5 and P6 (Table 1) and determined their nucleotide sequences. Inasmuch as all 10 clones displayed exactly the same DNA sequence, which was identical to the sequence deposited in GenBank (accession number M35008), we confirmed that marker D detects only a single, unique site within the CHO cell *RPS14* locus.

Taken together, the data obtained with all four STSs located a chromosomal OBR between *RPS14* nucleotides 3410 and 5400 (initiation zone in Fig. 4). This analysis, therefore, closely agrees with the conclusion obtained by examination of Okazaki fragment distributions (Fig. 2 and 3). Both biochemical analyses indicated that an OBR, hereafter designated ori_{S14}, is located within the 3′ half of the CHO *RPS14* locus.

Protein binding sites and other structural features of ori_{S14}. Inspection of the nucleic acid sequence encoding ori_{S14} reveals several interesting features. ori_{S14} includes an unusually large number of binding sites for well-known transcription and replication factors as well as other growth-regulating proteins.

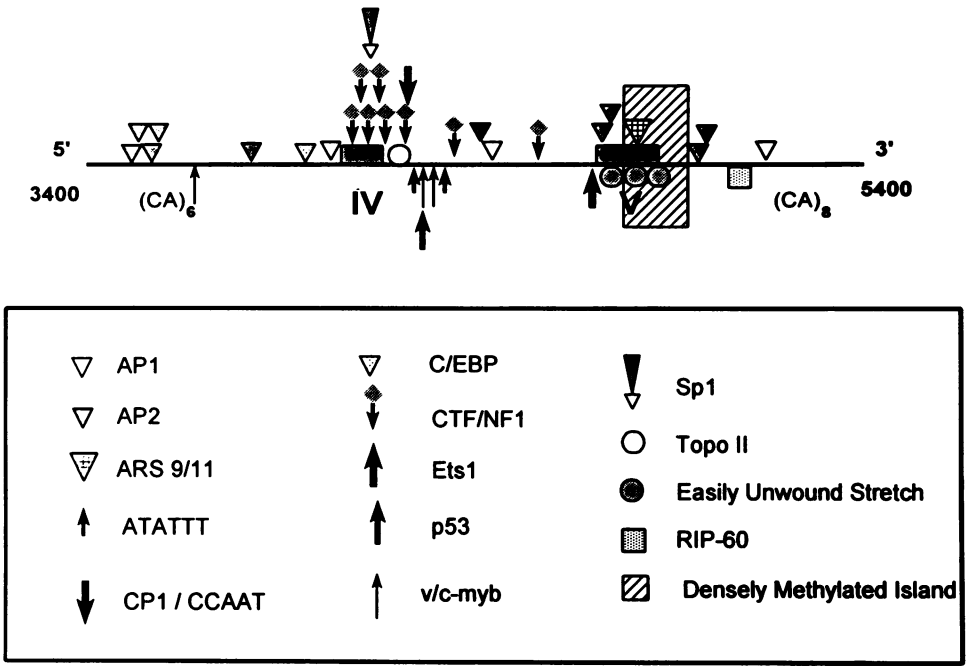

FIG. 5. CHO *RPS14* replication origin. ori_{S14} contains numerous binding sites for mammalian transcription factors, replication proteins, and oncogenes as well as sequence motifs attributed to other eukaryotic replication origins. The 2-kbp CHO *RPS14* OBR (nucleotides 3400 and 5400) is diagrammed to indicate the locations of protein binding sites and other structural features recognized (see text for details). Exons IV and V are represented as shaded rectangles labeled IV and V. The locations of two extended dinucleotide repeats, $(CA)_6$ and $(CA)_8$, also are indicated. Symbols used in the diagram are identified below the map. Topo, topoisomerase.

In addition, ori_{S14} contains several dinucleotide repeat sequences and, as described in the accompanying report (44), an unusual 127-bp sequence motif in which every dC is methylated when the cells are actively replicating chromosomal DNA. These features are diagrammed in Fig. 5.

A computer-based survey of the ori_{S14} DNA sequence versus the Transcription Factor Database (21) uncovered multiple potential binding sites for transcription factors AP1, AP2, and NF1 as well as single binding sites for CP1, C/EBP, and Sp1. It also detected consensus DNA recognition sites for the mammalian oncogenes encoding Ets1, p53, and Myb. Inspection of ori_{S14} also indicated DNA target sequences for two mammalian replication proteins: RIP-60, which binds to bent DNA sites within the CHO *ori*-β replication origin (8), and topoisomerase II (41). In addition, ori_{S14} harbors several extended, easily unwound, AT-rich sequences, two $(CA)_n$ repeat motifs, and a DNA motif which matches the consensus yeast autonomously replicating sequence at 9 of 11 positions (18). In all of these respects ori_{S14} resembles other mammalian replication origins whose base sequences are known (13, 20, 51).

DISCUSSION

Two experimental approaches were used to recognize an OBR within or adjacent to the Chinese hamster *RPS14* locus on chromosome 2q. The first approach involved isolation of discontinuous DNA replication intermediates (Okazaki fragments) from CHO cells presynchronized to early S phase. Okazaki fragment probes, labeled in vitro with ^{32}P and BrdU, detected the retrograde sides of replication forks traversing the *RPS14* locus in vivo. Because a pair of replication forks diverges from each eukaryotic chromosomal replication origin (12), one expects that chromosome origins will be associated with abrupt transitions in the DNA template strand complementary to Okazaki fragments, i.e., their retrograde strands (40). Our data indicate that such a transition occurs within or immediately downstream of the transcribed portion of the CHO *RPS14* locus (Fig. 2 and 3). The site of this transition, designated ori_{S14} maps between intron 3 and the proximal downstream flanking sequence (nucleotide residues 3510 to 5140). Further, our experiments demonstrate that cultured CHO cells initiate DNA replication at ori_{S14} during the initial five minutes of S phase. Since *RPS14* is a constitutively expressed housekeeping gene (36, 38), this replication program is consistent with the general correlation between transcriptionally active genes and early-replicating DNA in mammalian cells (16, 17, 23).

The second experimental approach used to identify an OBR within the CHO *RPS14* locus was based on the idea that unique chromosomal markers near a replication origin are contained within newly replicated DNAs of all sizes, whereas markers distal to a replication origin are contained only in longer nascent DNAs. This strategy, as first implemented by Vassilev et al. (47, 49) and then modified by Virta-Pearlman et al. (51), obtains its sensitivity through the use of PCR to detect gene-specific chromosomal STSs within size-fractionated, BrdU-labeled nascent DNA. As illustrated in Fig. 4, this approach also detected an OBR within *RPS14* and located the origin (ori_{S14}) within the 3′ half of the gene (nucleotide residues 3410 to 5400).

An unresolved, probably methodological paradox continues to confound molecular analyses of chromosomal replication origins in eukaryotic organisms. Biochemical methods reproducibly map mammalian chromosomal origins to single chromosome sites within relatively short regions of cloned genomic DNA (1 to 2 kbp). In contrast, physical mapping methods

based on two-dimensional gel electrophoresis and electron microscopy consistently indicate that the same replication origins are composed of multiple, closely spaced initiation sites arrayed over much longer (50- to 70-kbp) genome segments (14, 15, 29). In agreement with biochemical experiments performed by numerous laboratories on several other mammalian chromosomal origins (1, 6, 7, 10, 20, 22, 49, 51), our data map ori_{S14} to a relatively narrow initiation zone (~2 kbp) on CHO chromosome 2q. Whether a much larger, more complicated initiation zone might be revealed at this locus by electrophoretic mapping procedures remains to be determined.

ori_{S14} exhibits an interesting primary structure, as it includes a large number of potential binding sites for well-studied transcription factors, replication proteins, and mammalian oncogenes, as well as an AT-rich, easily unwound DNA sequence, two $(CA)_n$ dinucleotide repeats, and an oligo(dA) DNA bending sequence (Fig. 5). Although it is attractive to speculate that some or all of these motifs might play important roles in regulating ori_{S14}, experiments to test these hypotheses have only recently been initiated. Nonetheless, the unusual density of presumptive DNA regulatory motifs within the DNA sequence spanned by ori_{S14} is yet another feature shared among the few metazoan chromosomal replication origins whose nucleotide sequences are known.

ori_{S14} displays two characteristics that distinguish it from most other cloned mammalian replication origins. First, the ori_{S14} replication initiation zone substantially overlaps a transcriptionally active gene sequence. Other higher eukaryotic OBRs recognized so far are located in nontranscribed, intergenic chromosomal sequences (1, 5, 6, 22, 48, 51). Second, because *RPS14* maps to a large hemizygous segment of chromosome 2q (52), CHO-K1 cells contain only a single copy of ori_{S14}. Other available mammalian replication origins are dizygous autosomal loci which are present in two copies per diploid somatic cell. Inasmuch as biochemical and genetic studies are facilitated through use of single-copy chromosome markers, ori_{S14} is uniquely suited for detailed molecular genetic analysis using these approaches.

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