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Distal Sequences, but Not *ori*-β/OBR-1, Are Essential for Initiation of DNA Replication in the Chinese Hamster *DHFR* Origin

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Summary

In the Chinese hamster dihydrofolate reductase replication initiation zone, the *ori*-β locus is preferred over other start sites. To test the hypothesis that *ori*-β contains a genetic replicator, we restored a deletion in the 3′ end of the *DHFR* gene with a cosmid that provides the missing sequence and simultaneously knocks out the downstream *ori*-β locus. Replication initiates normally in *ori*-β knockout cell lines, and the *DHFR* domain is still synthesized in early S phase. However, initiation is completely suppressed in the starting deletion variant lacking the 3′ end of the gene. We conclude that *ori*-β does not contain an essential replicator, but that distant sequence elements have profound effects on origin activity in this locus.

Introduction

In bacterial, plasmid, viral, and yeast replicons, initiation is controlled by the interaction of an initiator protein or complex with a defined genetic element known as a replicator (Jacob and Brenner, 1963; reviewed in Kornberg and Baker, 1992). In these systems, replicators were identified by selecting for sequences capable of directing autonomous replication of collinear markers. Since nascent strand start sites approximately colocalize with the replicators in these systems, the general term *origin* is often used to refer to both the replicator and the initiation zone surrounding it.

However, autonomously replicating sequence (ARS) assays have not been successful in identifying elements in higher eukaryotic chromosomes that behave as bona fide replicators. In fact, when a nuclear partition function is provided in the cloning vehicle, virtually any human genomic sequence of sufficient length can replicate extrachromosomally for extended time periods (Krysan et al., 1989; Heinzel et al., 1991). A similar result is obtained when random DNA sequences are injected into *Xenopus laevis* oocytes or are incubated in egg extracts that support in vitro replication (Harland and Laskey, 1980; Blow and Laskey, 1986; reviewed in Blow et al., 1987).

Thus, several different methods have been developed to localize nascent strand start sites in a chromosomal region of interest, with the expectation that they will lie close to required genetic elements. The *DHFR* domain in CHO cells, which is the subject of the present report, has been analyzed by almost all of the available origin mapping methods (reviewed in DePamphilis, 1993; Burhans and Huberman, 1994; Hamlin and Dijkwel, 1995; Heintz, 1996). Several intrinsic labeling strategies have shown that replication initiates in the 55 kb spacer region between the *DHFR* and *2BE2121* genes, with two subregions (termed *ori*-β and *ori*-γ) being preferred (Figure 1A; Heintz and Hamlin, 1982, 1983; Anachkova and Hamlin, 1989; Handeli et al., 1989; Leu and Hamlin, 1989; Wang et al., 1998; S. Wang et al., submitted). *Ori*-β and *ori*-γ lie ~22 kb apart and straddle a matrix attachment region (MAR in Figure 1A; Dijkwel and Hamlin, 1988). Additional approaches focused only on the region encompassing *ori*-β and concluded that it constitutes a major start site in this locus (Burhans et al., 1986, 1990; Vassilev et al., 1990; Pelizon et al., 1996). Thus, *ori*-β was designated the origin of bidirectional replication (OBR-1) for the *DHFR* locus (Burhans et al., 1990).

In a very recent study, a sensitive nascent strand size and abundance assay was used to examine the left half of the intergenic spacer and detected not only *ori*-β, but an additional, less prominent, peak of small nascent strands ~5 kb downstream from *ori*-β (termed *ori*-β′; Kobayashi et al., 1998). These findings raised the possibility that *ori*-β, *ori*-β′, and/or *ori*-γ might coincide with classic genetic replicators, with the majority of initiations occurring within the immediate vicinity of these elements. However, neutral/neutral (Brewer and Fangman, 1987) and neutral/alkaline (Nawotka and Huberman, 1988) 2-D gel methods have shown that initiation occurs in every restriction fragment that was examined in the 55 kb intergenic spacer, with the 30 kb region encompassing *ori*-β and *ori*-γ being preferred (Vaughn et al., 1990; Dijkwel and Hamlin, 1992, 1995; Dijkwel et al., 1994).

We believe that these data are most compatible with a model in which the intergenic region corresponds to a broad zone of potential initiation sites, some of which are preferred. To determine whether an initiation reaction with these characteristics is controlled by classic replicators, we have developed a novel reach out and knock out (ROKO) strategy for mutagenizing sequences in the intergenic region, in which a donor cosmid from the *DHFR* locus is used to restore the 3′ end of a truncated, nonfunctional *DHFR* gene while simultaneously replacing the downstream target with a *neo*^{r} marker (X. L., unpublished data). In this study, we have used this strategy to delete a 4.3 kb XbaI fragment containing *ori*-β, since all methods of analysis are consistent with the suggestion that *ori*-β is the most preferred region or site for initiation in the *DHFR* locus. Two-dimensional gel analysis shows that replacement or complete removal of *ori*-β has no overt effect on either the efficiency of initiation or timing of origin activity in the *DHFR* locus. Importantly, however, origin activity is completely abolished in the starting cell line that lacks the 3′ end of the *DHFR* gene. The implications of these findings are discussed.

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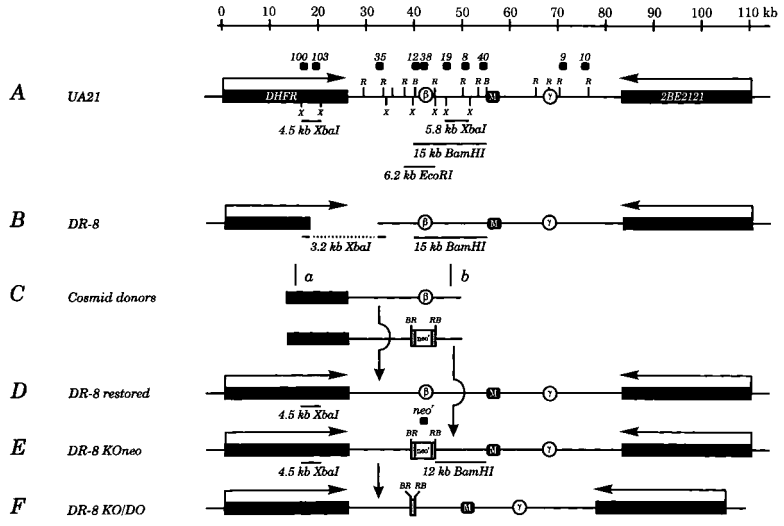

Figure 1. Maps of the *DHFR* Locus in Wild-Type and Knockout Cell Lines

(A) Map of the central 110 kb region encompassing the convergently transcribed *DHFR* and *2BE2121* genes, *ori*-β, *ori*-γ, and the matrix attachment region (MAR; closed rectangle) in the hemizygous wild-type *DHFR* locus in UA21 cells. Relevant EcoRI (R), XbaI (X), and BamHI (B) sites are indicated. Diagnostic restriction fragments and relevant hybridization probes are shown below and above the map, respectively.

(B) Map of DR-8, the starting DHFR-deficient deletion mutant used as the recipient for restoration of the *DHFR* gene and replacement of *ori*-β.

(C) The cosmid donor, KZ381, which was used to restore the *DHFR* gene, and a derivative (KZ381*neo*) in which the 4.3 kb XbaI fragment containing *ori*-β was replaced with the *neo*^{r} cassette (see text).

(D) The DHFR-positive restored product of homologous recombination between DR-8 and the cosmid, KZ381.

(E) A DHFR-positive restored knockout cell line (DR-8 *KOneo*) in which *ori*-β has been replaced with the *neo*^{r} marker donated by KZ381*neo*.

(F) The *KO/DO* derivative of DR-8 KO*neoR* from which the *neo*^{r} cassette was removed by the action of *cre* recombinase.

Results

The Reach Out and Knock Out Recombination and Selection Strategy for Mutagenizing Sequences in the Intergenic Spacer

The ROKO approach begins with a DHFR-deficient cell line (DR-8) that has deleted a 13.5 kb region encompassing the 3′ end of the *DHFR* gene from the hemizygote, UA21 (Urlaub and Chasin, 1980; Jin et al., 1995; Figure 1B). Thus, DR-8 can be propagated on a medium that supplies thymidine, hypoxanthine, and glycine (e.g., F12) but cannot survive on medium lacking these components (e.g., F12-special; Urlaub and Chasin, 1980; Urlaub et al., 1983; Jin et al., 1995). We reasoned that KZ381, a cosmid whose insert extends from the middle of the gene to the 3′ side of *ori*-β (Ma et al., 1990), should be able to restore the *DHFR* gene by homologous recombination and, thus, allow growth of recombinants on F12-special (Figures 1C and 1D). If *ori*-β were replaced in the cosmid with a *neo*^{r} marker, crossovers occurring near positions a and b should simultaneously restore the gene and knock out *ori*-β (Figures 1C and 1E). Because cosmid KZ381 does not contain the 5′ half of the gene, it cannot itself restore DHFR activity to a DHFR-deficient cell line by integration at an ectopic site (Y. Shan and J. L. H., unpublished data).

We first tested the ability of cosmid KZ381 to restore DHFR activity to DR-8. DR-8 cells were electroporated with KZ381, and homologous recombinants were selected on F12-special. An analysis of one of the successful recombinants (DR-8 restored) is presented in Figure 2. Hybridization of an XbaI genomic digest with probe 100 (Figure 1A) shows that the 3.2 kb XbaI fragment that straddles the deletion junction in DR-8 is restored to the 4.5 kb XbaI fragment characteristic of the wild-type *DHFR* gene (Figure 2B; compare DR-8 restored to the UA21 and CHO patterns). Diagnostic EcoRI, BamHI, and HindIII digests confirmed the successful restoration of the gene (data not shown). As expected, the *ori*-β region appears not to be affected by the recombination event that restored the 3′ end of the gene (Figures 2A and 2C): probes 40+8, which hybridize to a 15 kb BamHI

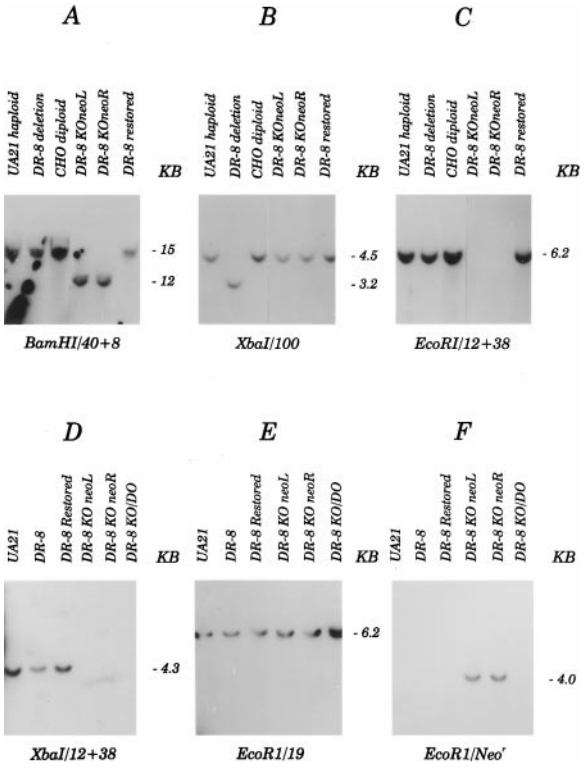

Figure 2. Characterization of Knockout Derivatives of DR-8

BamHI, XbaI, and EcoRI digests of DNA from the indicated cell lines were transferred to HybondN$^+$ and hybridized with diagnostic probes to detect homologous recombinants and removal of the *neo*^{r} marker by the action of the *cre* recombinase (see Figure 1A for probe positions and fragment sizes).

fragment in UA21 and CHO cells, detect a similar-sized fragment in the restored variant (Figure 2A), and probes 12+38 detect the wild-type 6.2 kb EcoRI fragment that contains *ori*-β (Figure 2C).

The Downstream Origin Is Active in the Restored Cell Line, but Not in DR-8, which Lacks the 3′ End of the *DHFR* Gene

To confirm that origin activity was intact in the starting DR-8 cell line lacking the 3′ end of the gene, we compared its replication pattern to that of the wild-type hemizygote, UA21, using neutral/neutral 2-D gel analysis (Brewer and Fangman, 1987; see Figure 3, lower panels, and legend for principle of the method). DR-8 and UA21 cells were synchronized with mimosine at the G_1/S boundary as described in Experimental Procedures (Mosca et al., 1992), and mimosine was removed to allow entry into the S period after a lag of ~50 min. Samples were taken 80, 160, and 360 min later, and replication intermediates were isolated and digested with EcoRI. After separation on a 2-D gel and transfer to a membrane, the digests were hybridized with probes 12+38, which are specific for the 6.2 kb fragment containing *ori*-β.

The pattern of replication intermediates in the wild-type UA21 control (Figure 3, upper row) is typical of that observed in CHO (Dijkwel and Hamlin, 1995) and CHOC 400 cells (Vaughn et al., 1990; Dijkwel and Hamlin, 1992; Dijkwel et al., 1994). At the 80 min time point when initiation is maximal in this locus, a composite pattern is detected in the *ori*-β-containing fragment that consists of a complete bubble arc and a more pronounced single fork arc. The bubble arc corresponds to initiations occurring within the fragment. The single fork arc must arise from initiations that occurred in neighboring fragments within the intergenic zone itself, since very few replication forks have advanced out of the intergenic region by 80 min after mimosine removal (Dijkwel and Hamlin, 1992, 1995; Dijkwel et al., 1994). By 160 min (Figure 3B, upper row), the bubble arc has almost disappeared from the *ori*-β-containing fragment, but the single fork arc persists, as it does in the 360 min sample (Figure 3C). This is the consequence of the fact that only some copies of this origin fire in any given S period, while those that do not fire are replicated passively much later by forks emanating from a distant upstream or downstream origin in another replicon (Dijkwel et al., 1994; Dijkwel and Hamlin, 1995). Probe 19, which is specific for the 6.1 kb fragment to the right of *ori*-β, reveals a very similar pattern at each time point (data not shown; Dijkwel and Hamlin, 1992, 1995; Dijkwel et al., 1994).

Surprisingly, in the deletion variant, DR-8, early-firing origin activity in the intergenic spacer is almost completely eliminated (Figure 3, second row). Virtually no replication intermediates of any kind can be detected in the 80 or 160 min samples in the fragment containing *ori*-β (probed with 12+38) or in the neighboring 6.1 kb fragment to the right (hybridized with probe 19; data not shown). A fragment from the *ori*-γ locus also is devoid of replication intermediates at these time points (data not shown). By 360 min, a single fork arc appears in *ori*-β, but no bubble arc is visible (Figure 3C). When the same blot was stripped and rehybridized with a probe for a 6.5 kb EcoRI fragment from the early-firing rhodopsin origin (Gale et al., 1992), the resulting patterns were very similar to those of the *DHFR* locus in UA21 cells at the same time points (upper panels). Thus, the failure to detect initiation in the *ori*-β region in DR-8 cells in early S phase cannot be attributed to a lack of cell synchrony or inadvertent loss of replication intermediates.

When the 13.5 kb deletion in DR-8 is restored by

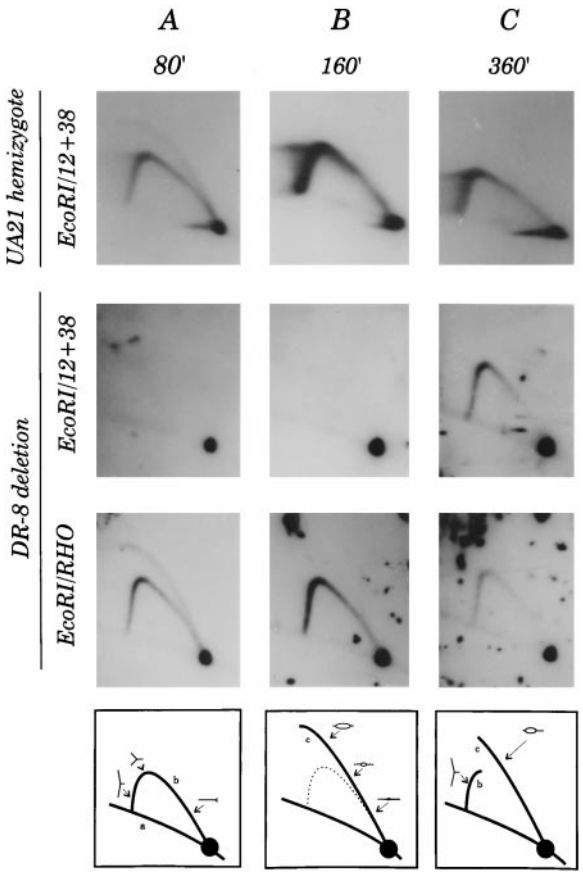

Figure 3. Deletion of the 3′ End of the *DHFR* Gene Results in Loss of Origin Activity in the Intergenic Region

(Upper three rows) The hemizygous UA21 control and the DHFR-deficient DR-8 derivative were synchronized as described in the text, and samples were collected 80, 160, and 360 min after release from mimosine. Replication intermediates were digested with EcoRI, separated on a neutral/neutral 2-D gel, and transferred to a membrane. The membranes were hybridized with a combination of probes 12 and 38, which are specific for the 6.2 kb EcoRI fragment containing *ori*-β (see Figure 1A). The blots were then stripped and reprobed with a 4.8 kb EcoRI fragment from the early-firing rhodopsin origin. (Bottom row) Principle of the neutral/neutral 2-D gel replicon mapping technique (Brewer and Fangman, 1987). Curve a represents the diagonal of linear, nonreplicating fragments in the genome. Curve b represents the single fork or simple Y arc characteristic of a passively replicated fragment. Curve c corresponds to the arc of bubble-containing fragments. A complete bubble arc is obtained when a fixed origin is centered in a fragment, as shown in the middle panel, or when initiation occurs at any one of several sites distributed throughout the fragment, some of which are centered (Vaughn et al., 1990; Dijkwel et al., 1994; Dijkwel and Hamlin, 1995). The panel on the right shows the pattern obtained when a fixed origin is off center in a fragment.

homologous recombination (Figure 4, upper two rows), initiation in the intergenic region appears to be fully reactivated in the early S period (90 min), as indicated by the presence of both bubbles and single fork arcs in the *ori*-β-containing fragment (probed with 12+38) and in the adjacent 6.1 kb EcoRI fragment to the right (probe 19). Therefore, since the same cosmid that corrects the initiation defect in DR-8 was used as the donor to prepare the *ori*-β knockout cell lines, any defect in replication in the knockout cell lines must have resulted from loss of *ori*-β and not some other mutation in the DR-8 cell line.

Deletion of the *ori*-β Region by the ROKO Approach

To test the possibility that *ori*-β contains an essential genetic replicator, a knockout donor cosmid was constructed in which a 4.0 kb *neo*^{r} cassette was cloned in either orientation in place of the 4.3 kb XbaI fragment that contains *ori*-β/OBR-1 (Figures 1C and 1E; the rightward and leftward transcribing versions of the cosmid are termed *neoR* and *neoL*, respectively). The *neo*^{r} cassette in the donor cosmid was flanked with *loxP* sites to allow subsequent removal from homologous recombinants by cleavage with the *cre* recombinase (Sauer and Henderson, 1988). DR-8 cells were electroporated with either the *neoR* or *neoL* cosmid donor, and potential homologous recombinants were selected first on F12-special medium and then on G418. Hybridization analysis of successful homologous recombinants is shown in Figures 2A–2C, in which BamHI, XbaI, and EcoRI digests of DNA from DR-8 *KOneoL* and DR-8 *KOneoR* revealed the predicted fragment sizes when hybridized with probes 40+8, 100, and 12+38, respectively (see Figure 1 for diagnostic fragments and probes). Fluorescence in situ hybridization was used to confirm that the DR-8 restored, DR-8 *neoL*, and DR-8 *neoR* cell lines had integrated the transfected donor cosmids exclusively at the native *DHFR* locus on the long arm of chromosome Z2 (data not shown).

Replacement or Removal of ori-β Has No Detectable Effect on Early-Firing Origin Activity in the Intergenic Locus

To assess the effects of replacing the 4.3 kb XbaI fragment that contains *ori*-β with the *neo*^{r} cassette, replication intermediates from the DR-8 *KOneoL* and DR-8 *KOneoR* cell lines were analyzed on a neutral/neutral 2-D gel, using probe 19 to detect the 6.1 kb EcoRI fragment that lies immediately to the right of the *ori*-β-containing fragment (see Figure 1A). As shown in Figure 4 (third and fourth rows), the replacement of *ori*-β with the *neo*^{r} marker has no dramatic effect on early origin activity in either of the knockout cell lines (DR-8 *KOneoL* and DR-8 *KOneoR*): in both cases, the typical composite pattern consisting of a bubble arc as well as a single fork arc appears at 80 min, and the intensities relative to the 1*n* spot are similar to those of the DR-8 restored control and the wild-type hemizygote, UA21 (Figures 3 and 4).

Similar results were obtained in both knockout cell lines with probe 35, which hybridizes to a 4.1 kb EcoRI fragment lying ~8 kb upstream from the usual position of *ori*-β (Figure 1A). The results for probe 35 on the DR-8 *neoL* derivative are compared to those with probe 19 in Figure 4 (fourth and fifth rows, respectively). Therefore, fragments both upstream and downstream from the *neo*^{r} gene are able to initiate efficiently regardless of the orientation of the gene. Interestingly, we were not able to detect bubble arcs within the *neo*^{r} marker that replaces *ori*-β, even though the single fork arc is as intense as that detected with probe 35 (Figure 4, lower panels).

To eliminate the possibility that transcriptional activity

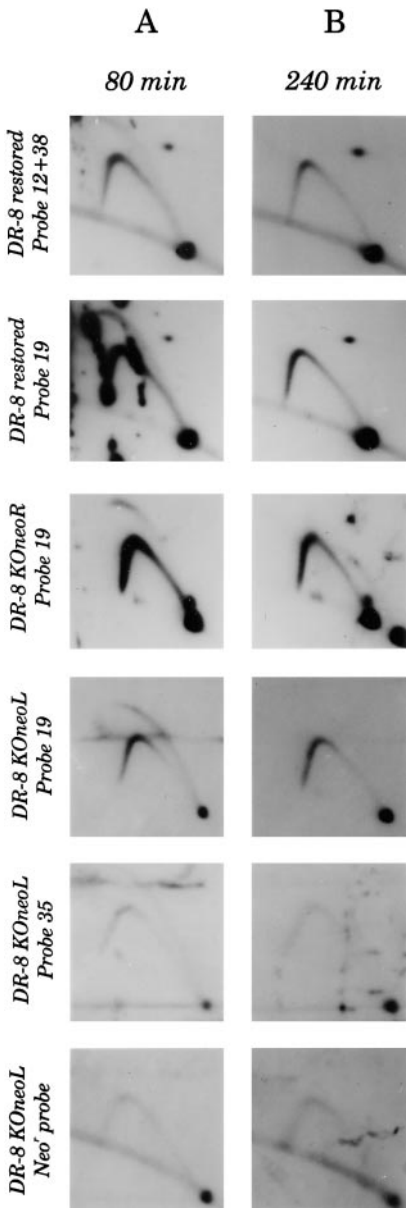

Figure 4. Replacement of ori-β with a *neo*^{r} Marker Has No Apparent Effect on Initiation in the Remainder of the Intergenic Region

The DR-8 derivative in which the *DHFR* gene had been restored (DR-8 restored), as well as the two derivatives in which *ori*-β had been replaced with the *neo*^{r} marker in opposite orientations, were synchronized as described, and samples were taken for neutral/neutral 2-D gel analysis 80 and 240 min after release from mimosine. Replication intermediates were digested with EcoRI, separated on a neutral/neutral 2-D gel, and hybridized with the indicated probes.

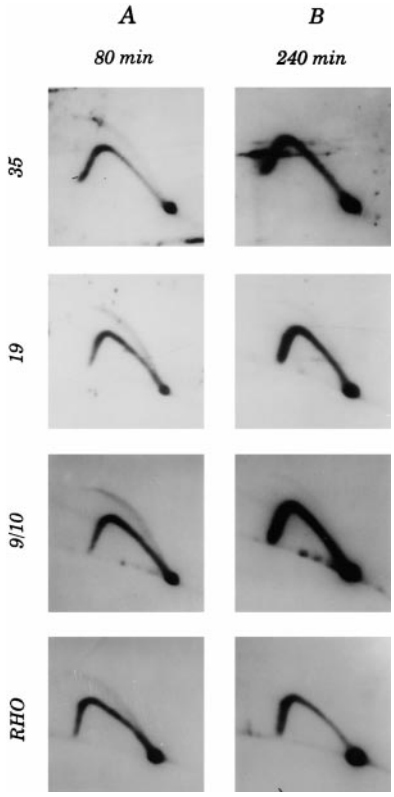

Figure 5. Removal of the *neo*^{r} Cassette from DR-8 KOneoR Has No Apparent Effect on Origin Activity in the Intergenic Region

DR-8 *KO/DO* cells were synchronized at the G_1/S boundary with mimosine as described, and samples were harvested 80 and 240 min after removal of mimosine. Replication intermediates were digested with EcoRI, separated on a neutral/neutral 2-D gel, and hybridized successively with the indicated probes.

in the *neo*^{r} marker could somehow compensate for the loss of a replicator in the *ori*-β knockout, the *neo*^{r} cassette was deleted from the DR-8 *KOneoR* cell line by *cre*-mediated cleavage and rejoining at the flanking *loxP* sites (Sauer and Henderson, 1988). Southern blotting and hybridization data for one of these clones are shown in Figures 2D–2F. In XbaI and EcoRI digests, the expected patterns were obtained when hybridized with probes specific for the *ori*-β region (probes 12+38, Figure 2D), for the neighboring 6.1 kb EcoRI fragment (probe 19, Figure 2E), or for the *neo*^{r} marker (Figure 2F).

When this *ori*-β drop-out cell line (DR-8 *KO/DO*) was analyzed on a neutral/neutral 2-D gel (Figure 5), the pattern of initiation in the intergenic spacer was indistinguishable from that of the wild-type locus in UA21 cells (compare to upper panels in Figure 3 and to the rhodopsin origin in the lower panels in Figure 5A). Eighty minutes after release from mimosine, bubble arcs were detected in the 4.1, 6.1, and 5.9 kb EcoRI fragments with probes 35, 19, and 9+10, respectively (Figure 5A). By 240 min, these fragments are largely populated by single forks, with only a low level of replication bubbles detectable (Figure 5B; note that the film exposures for the 240 min samples are more intense than those for the 80 min samples; compare 1*n* spots).

Neutral/Alkaline 2-D Gel Analysis and a Replication Timing Assay Suggest that the *DHFR* Domain Is Synthesized at the Normal Time by Forks that Initiate in the Intergenic Zone

It was important to determine whether replication forks in the DR-8 *KO/DO* cell line originate in and move away from sites in the remainder of the intergenic region at approximately the same time in the S period as in the wild-type locus. Therefore, we examined the direction and timing of replication fork movement through the intergenic region and the *DHFR* gene using the neutral/alkaline 2-D gel method (Figure 6D; Nawotka and Huberman, 1988; see figure legend for details). The DR-8 *KO/DO* cell line was synchronized as described, and samples were taken 80 and 240 min after removal of mimosine. Replication intermediates were digested with XbaI and separated on a neutral/alkaline 2-D gel. The digest was hybridized successively with probes 19 and 8, which are specific for the 5′ and 3′ ends of the 5.8 kb XbaI fragment to the right of the *ori*-β locus in the intergenic spacer (Figure 1A). As shown in Figure 6A, each of these probes detects a complete diagonal in the 80 min sample, indicating that forks are entering this fragment from both upstream and downstream directions at this time point. This is the pattern expected of a fragment residing in a broad initiation zone (Vaughn et al., 1990; Dijkwel and Hamlin, 1992).

The transfer was then hybridized successively with probes 100 and 103 for the 5′ and 3′ ends of a 4.5 kb fragment from the body of the *DHFR* gene (Figure 1A). As in the wild-type locus (Dijkwel et al., 1994), very few replication forks have reached the gene from the intergenic spacer by 80 min, with the consequence that neither the 5′ nor 3′ probe detects significant numbers of replication intermediates (Figure 6B). Therefore, the forks detected in the 5.8 kb XbaI fragment in the intergenic spacer at this time point must have arisen from initiation sites within the spacer region itself and not from upstream or downstream replicons. By 240 min after removal of mimosine, a strong diagonal is illuminated with the origin-proximal 3′ probe, while only the largest nascent strands hybridize with the origin-distal 5′ probe (Figure 6C). Since significant numbers of forks are not detected in the intergenic region until 360 min after release from mimosine in the starting DR-8 deletion (Figure 3; P. A. D., unpublished data), we conclude that the forks detected in the *DHFR* gene at 240 min in the *ori*-β drop-out cell line must have arisen from the intergenic region itself. These data are virtually identical to the results of neutral/alkaline 2-D gel analysis on the wild-type locus in CHO cells (Dijkwel and Hamlin, 1995).

We also determined the time of replication of the *DHFR* domain in the *ori*-β knockout cell line, DR-8 *KO/DO*, by an independent fluorescence in situ hybridization technique (Kitsberg et al., 1993). Cells in an unsynchronized cell population were immobilized on a microscope slide, and the number of copies of the *DHFR* locus in different interphase nuclei (which doubles after the locus has been replicated) was determined by hybridization with a specific fluorescent probe. Comparison to the early-replicating rhodopsin locus in the same cell indicates whether the *DHFR* locus is early or late replicating. Rhodopsin-specific (C3B) and *DHFR*-specific (KD504)

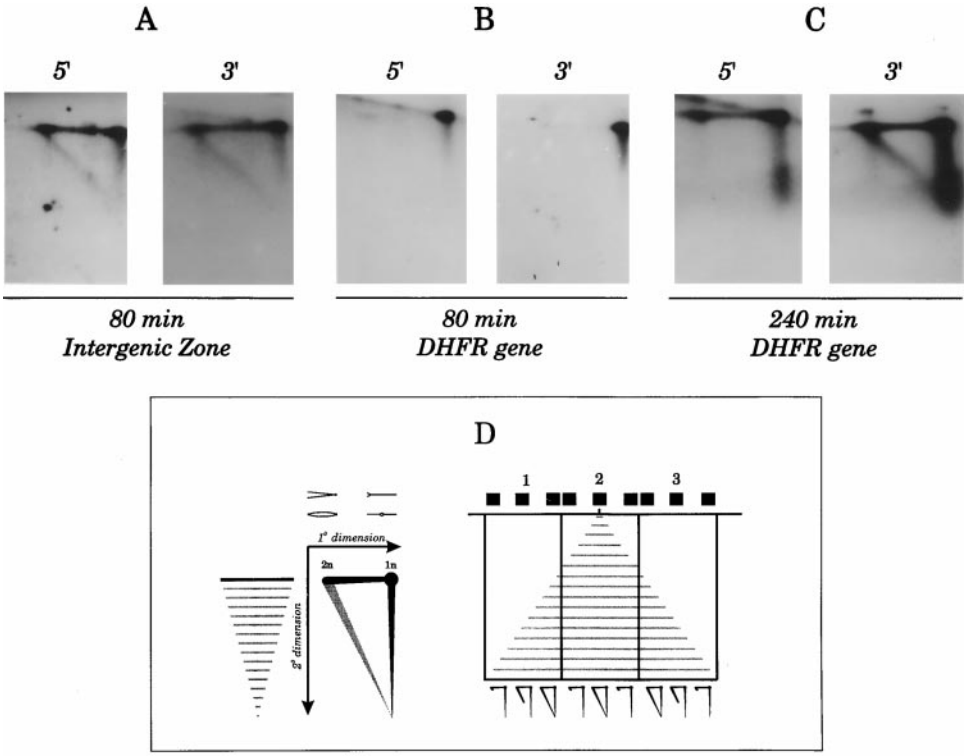

Figure 6. Replication Forks in the DR-8 KO/DO Cell Line Arise in and Move Away from the Intergenic Region with the Same Kinetics as in the Wild-Type Locus

(A–C) The DR-8 *KO/DO* cell line was synchronized at the G_1/S boundary, and samples were harvested 80 and 240 min after removal of mimosine. Replication intermediates were prepared for neutral/alkaline 2-D gel analysis, using XbaI to digest the DNA. After transfer to a membrane, the digests were hybridized successively with probes 19 and 8 for the ends of the 5.8 kb XbaI fragment in the intergenic region, and then with probes 100 and 103 for the 6.5 kb fragment in the body of the *DHFR* gene.

(D) Principle of the neutral/alkaline 2-D gel replicon mapping method (Nawotka and Huberman, 1988). On the left is shown the separation of the partially double-stranded replication intermediates in the first dimension and the single-stranded template (horizontal black band) and nascent strands (gray diagonal) in the second dimension. On the right are shown three restriction fragments encompassing a fixed origin, as well as the patterns of hybridization that would be obtained by probes from the ends or the center of each fragment.

cosmids were labeled with digoxigenin- and biotin-labeled dUTP, respectively, and the combination was hybridized to asynchronous populations of parental UA21 cells, the DR-8 deletion variant, the DR-8 restored cell line, and DR-8 *KO/DO*. After hybridization, the rhodopsin-specific cosmid was detected with Texas red–labeled anti-digoxigenin (represented by the open circles in Figure 7) and the *DHFR*-specific probe with fluorescein-labeled avidin (closed circles). The six possible patterns of hybridization are illustrated in panels (A) through (F), where (A) corresponds to the unreplicated state (G_1 and early S phase prior to the time of replication of the particular locus), and (B) through (F) correspond to various stages of replication of the rhodopsin and *DHFR* loci.

As indicated in Figure 7, 85% of S phase UA21 control cells in this sample have replicated the rhodopsin domain (column H), while 77% have replicated the *DHFR* locus. Thus, both appear to be synthesized during approximately the same interval in early S phase. For S phase DR-8 cells, however, 95% have replicated at least one rhodopsin locus and only 48% have replicated *DHFR*, confirming that the *DHFR* domain has become late replicating in this deletion variant. (Note that, even if the *DHFR* origin is completely inactive, this number will never go to zero because many of the cells shown in (F) represent late S phase and G_2 cells in which even a very late-replicating *DHFR* locus will already have doubled. Thus, this calculation underestimates the difference in replication timing between any two loci. However, if the late S and G_2 cells in (F) are excluded from the calculations, then 95% of the remaining S phase

	A	B	C	D	E	F	G	H	I
RHO / *DHFR*	○ / ●	○○ / ●	○○ / ●	○ / ●●	○○ / ●●	○○ / ●●	B-F	B,C,E,F / G	D,E,F / G
UA21	32	10	6	10	19	23	68	**85**	**77**
DR-8	25	20	19	4	10	22	75	**95**	**48**
DR-8/Res	33	10	4	9	21	23	67	**87**	**79**
DR-8 KO/DO	32	14	4	13	16	21	68	**81**	**74**

Figure 7. Determination of the Time of Replication of the *DHFR* Domain in the DR-8 Restored Cell Line

The cosmids C3B (rhodopsin-specific) and KD504 (*DHFR*-specific) were labeled, hybridized to the indicated cell types spread on microslides, and detected as described in Experimental Procedures. Fluorescent hybridization signals from 300, 435, 213, and 134 interphase nuclei in the UA21, DR-8, DR-8 restored, and DR-8 *KO/DO* cell lines, respectively, were scored and tabulated as shown (expressed as percentage of total).

cells have replicated rhodopsin and only 26% have replicated *DHFR*.) In the DR-8 restored cell line, 87% of S phase cells had already replicated the rhodopsin locus, and 79% had replicated the *DHFR* domain. Most importantly, the two values are very similar in the DR-8 *KO/DO* cell line as well (81% and 74%, respectively). Thus, it appears that removal of *ori*-β has no effect either on the efficiency of initiation or the time of replication of the *DHFR* locus.

Discussion

In plasmids, viruses, bacteria, and yeast, nascent chains initiate within narrowly circumscribed regions that invariably contain a genetic replicator (reviewed in Kornberg and Baker, 1992). With very few exceptions (e.g., Martin and Setlow, 1980; Tamanoi et al., 1980; von Meyenberg et al., 1987), deletion of the replicator inactivates origin function in the corresponding replicon. In mammalian genomes, there is some evidence that replication initiation zones can be very narrow (e.g., the human lamin B2 origin; Giacca et al., 1994), but many other higher eukaryotic replicons are characterized by broad initiation zones. Examples include the chorion (Delidakis and Kafatos, 1989; Heck and Spradling, 1990), α-polymerase (Shinomiya and Ina, 1994), and histone (Shinomiya and Ina, 1993) loci in *Drosophila melanogaster*; the II/9A puff region in *Rhynchosciara americana* (Liang et al., 1993); the rDNA locus in *Xenopus* (Hyrien et al., 1995); and the rDNA (Little et al., 1993), *DHFR ori*-α (Leu and Hamlin, 1992), *DHFR ori*-β/*ori*-γ (Vaughn et al., 1990; Dijkwel and Hamlin, 1992, 1995; Dijkwel et al., 1994), and rhodopsin (Gale et al., 1992; P. A. D., unpublished data) loci in mammalian cells.

To our knowledge, only two studies have provided good evidence for the existence of replicators in complex genomes. The first example is the chorion origin in *Drosophila*, which contains an amplification control element (ACE) that can seed a new zone of initiation when inserted into an ectopic chromosomal site by P element–mediated transduction (Delidakis and Kafatos, 1987, 1989; Heck and Spradling, 1990; but see Swimmer et al., 1989). In the human hemoglobin Lepore syndrome, an 8 kb deletion encompassing the 3′ end of one globin gene, the intergenic spacer, and the 5′ end of another globin gene results in loss of origin activity from this locus (Kitsberg et al., 1993). Furthermore, it has been demonstrated that the 8 kb region encompassing this origin can direct initiation from an ectopic site and that specific deletions affect origin activity (Aladjem et al., 1998).

In the present study, we directly addressed the question of whether the *ori*-β region, which is a preferred initiation locus in a broad zone of potential sites in the *DHFR* domain, contains a genetic replicator. We devised the reach out and knock out (ROKO) approach for constructing and identifying clones in which sequences in the vicinity of the *DHFR* gene have been replaced and then deleted. Neutral/neutral 2-D gel analysis of knockout clones prepared and selected by this strategy showed that replacement of the *ori*-β region with the *neo*^{r} marker in either orientation does not reduce origin activity in the remainder of the intergenic region (Figure 4). In fact, in DR-8 *neoL*, the 6.1 kb EcoRI fragment to the right of the *neo*^{r} marker displays the highest bubble-to-fork arc ratio that we have ever observed in any fragment from the initiation zone in any cell line. However, the same increase in initiation frequency was not detected in flanking fragments in the DR-8 *neoR* construct. This difference could result from a minor difference in cell synchrony if DR-8 *neoR* cells were inadvertently sampled after the peak initiation period. It could also reflect a real difference in the frequency of initiation depending upon whether transcription through the *neo*^{r} gene is pointed toward or away from this fragment. However, a fragment lying to the left of the *neo*^{r} marker in DR-8 *neoL* also sustains a high level of initiation (detected with probe 35, Figure 4A), arguing that the orientation of the marker does not significantly alter initiation frequencies in flanking regions.

Conceivably, the very presence of the *neo*^{r} transcription unit could be responsible for increased origin activity in flanking fragments: since the *neo*^{r} marker appears not to support initiation (Figure 4A, lower panels; H.-B. L., unpublished data), perhaps it effectively reduces the size of the initiation zones to its right and left in the intergenic spacer, thereby increasing the bubble-to-fork arc ratios. Alternatively, the deletion of *ori*-β may have eliminated interference among replicators at *ori*-β, *ori*-β′, and in the fragment upstream from *ori*-β, with the consequence that *ori*-β′ and the upstream replicator become much more efficient. This kind of origin interference has been observed to occur in neighboring origins in both *Saccharomyces cerevisiae* (Brewer and Fangman, 1993) and in *Schizosaccharomyces pombe* (Dubey et al., 1994). However, when the *neo*^{r} cassette was deleted by the action of the *cre* recombinase on the flanking *loxP* sites, the pattern of replication intermediates in the remainder of the intergenic region in the DR-8 *KO/DO* cell line was remarkably similar to the wild-type control (Figure 5), making origin interference unlikely in this case.

Neutral/alkaline 2-D gel analysis demonstrated that significant numbers of replication forks appear both in the intergenic region and in the *DHFR* gene from the direction of the intergenic region at about the same time in the *DR-8 KO/DO* cell line as in wild-type CHO cells (Figure 6). Furthermore, the replication timing assay indicated that the same percentage of *DR-8 KO/DO* cells replicate the *DHFR* locus in early S phase as do wild-type UA21 cells (Figure 7). Thus, the timing and efficiency of initiation in the intergenic region appear to be retained when *ori*-β is deleted.

Thus, we conclude that *ori*-β does not contain an essential genetic replicator. However, *ori*-β could represent a preferred replicator in a hierarchy of nonessential replicators distributed at frequent intervals throughout the intergenic region (or, indeed, the genome as a whole). Presumably, *ori*-β′, as well as a region just downstream from it, should contain such replicators, since they also have been suggested to correspond to highly preferred nascent strand start sites in this locus (Kobayashi et al., 1998). However, extensive analysis by neutral/neutral 2-D gel techniques has detected replication bubbles in every restriction fragment analyzed in the intergenic spacer, regardless of the enzyme used to digest the DNA (Vaughn et al., 1990; Dijkwel and

Hamlin, 1992, 1995; Dijkwel et al., 1994). In addition, variations of the 2-D gel techniques specifically designed to uncover a fixed initiation site near *ori*-β failed to do so (Kalejta and Hamlin, 1996; Kalejta et al., 1996). Thus, it is still not clear whether this initiation zone contains only a few relatively fixed sites, as suggested by the nascent strand size and abundance assay, or a large number of sites with some regions of the spacer being preferred, as suggested by 2-D gels. The latter model was suggested by Calos and coworkers to explain why any cloned mammalian DNA fragment can replicate autonomously in a mammalian cell background if it is sufficiently long (Krysan et al., 1989; Heinzel et al., 1991), and why replication appears to initiate at random sites within the inserts of these plasmids (Krysan and Calos, 1991).

We expected that the DR-8 cell line, which lacks the 3′ end of the *DHFR* gene but retains *ori*-β, would retain wild-type origin activity and therefore could serve as one of the positive controls for the *ori*-β knockout. Instead, this 13.5 kb deletion, which extends from the fifth intron of the gene into the intergenic region (Figure 1A), results in complete loss of early-firing origin activity in the remainder of the intergenic spacer (Figure 3). One explanation for this phenomenon is that the loss of 3′ processing signals allows the transcription complex to travel well into the intergenic region and interfere with the initiation and/or elongation machinery, which has been demonstrated to occur in *Escherichia coli oriC* (Tanaka and Hiraga, 1985), in the ColE1 origin (Stueber and Bujard, 1982), in yeast ARS elements (Snyder et al., 1988), and in cloned mammalian DNA fragments transfected into mammalian cells (Haase et al., 1994). A second possible explanation for loss of early-firing origin function in DR-8 is that a critical *cis*-regulatory element resides in the 13.5 kb deletion itself. In this case, the deletion must affect the entire chromosomal domain, since there are virtually no replication forks in the intergenic region in the DR-8 cell line 160 min after removal of mimosine (Figure 3B, middle panel), whereas large numbers of forks that presumably arose from upstream or downstream initiation sites are detected in the wild-type locus in UA21 cells at 160 min (Figure 3B, lower panel). For any of these scenarios, we cannot presently rule out the possibility that origin function has been delayed until very late in S phase in the DR-8 cell line. Indeed, because of decay of synchrony as cells traverse the S period, it would be very difficult to detect initiation events (i.e., bubbles) in late S phase if they were occurring. Finally, it is conceivable that some critical *trans*-acting factor is delivered to the intergenic region via the transcription complex and is released by normal 3′ processing events. All of these possibilities can be tested with variations of the ROKO approach by deleting selected sequences from the 3′ end of the wild-type gene.

Experimental Procedures

Construction of Donor Cosmid

A plasmid containing a 12 kb XhoI fragment from the *DHFR* intergenic region was partially digested with XbaI to liberate the 4.3 kb *ori*-β-containing subfragment, which was replaced with a 4.0 kb XbaI fragment from plasmid pLNLMCS2 (R. F. K., unpublished data) containing the *neo*^{r} marker flanked by *loxP* sites (Sauer and Henderson, 1988). This 12 kb *neo*^{r}-containing XhoI fragment was ligated into the single XhoI site of cosmid KZ381.

Cell Culture, Selection, and Synchronization

DR-8 cells were maintained in F12 medium supplemented with 10% Fetal Clone II (Hyclone) at 37°C in an atmosphere of 5% CO_2/95% air. DHFR$^+$ recombinants were selected in a special formulation of F12 (F12-special; Jin et al., 1995) that lacks thymidine, hypoxanthine, and glycine. F12-special was supplemented with 10% Fetal Clone I (Hyclone) and, where appropriate, 500 μg/ml G418 (GIBCO/BRL). Cells were synchronized in G_0 by starving for isoleucine for 37 hr and were released into drug-free medium containing 200 μM mimosine for 12 hr (Dijkwel and Hamlin, 1992). The medium was then replaced with drug-free complete medium to allow synchronous entry into the S period.

Transfection and Screening Protocol

Asynchronous cells were harvested by trypsinization and were washed and resuspended in electroporation buffer (20 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose [pH 7.05]) at a concentration of 2×10^7 cells/ml. Donor cosmids were linearized with SalI in the vector (pWE-16; Wahl et al., 1987), after which the reaction volume was adjusted to 400 μl with electroporation buffer. The solution was mixed with an equal volume of the cell preparation in a 0.4 cm cuvette on ice and subjected to a single pulse of 1000 volts (25 μF) with a Bio-Rad Gene Pulser. Cells were plated immediately in F12 medium, selection medium (F12-special) was applied 48 hr later, and surviving colonies were cloned into the wells of a 24-well dish (Costar) after 10–14 days. The DNA in DHFR$^+$ clones was analyzed by standard Southern blotting and hybridization procedures as described in the text.

Cre-Mediated Deletion of the *neo*^{r} Gene

The *cre* expression vector and the lipofection reagent, Lipofectamine, were purchased from Life Technologies, Inc. Approximately 5×10^5 DR-8 *KOneoR* cells were transfected with 1.5 μg *cre* expression plasmid and 6 μg Lipofectamine according to the supplier's recommendations. Twenty-four hours later, individual cells were cloned into the wells of 24-well dishes and grown to ~50% confluence before splitting and replating in duplicate in F12 medium ± 500 μg/ml G418. G418-sensitive cells were propagated and analyzed by Southern blotting to confirm the loss of the *neo*^{r} marker.

Replication Intermediate Isolation and 2-D Gel Electrophoresis

Replication intermediates were purified and separated either on neutral/neutral (Brewer and Fangman, 1987) or neutral/alkaline (Nawotka and Huberman, 1988) 2-D gels exactly as described previously (Dijkwel et al., 1991; Dijkwel and Hamlin, 1992). After the digests were transferred to Hybond N$^+$ membranes (Amersham), they were hybridized with the ^{32}P-labeled probes indicated in the figure legends (see probe positions in Figure 1A). Probes were as follows (see Figure 1A for positions): #100, a 1.2 kb XbaI/KpnI fragment; #103, a 1.2 kb EcoRI/XbaI fragment; #35, a 1.6 kb KpnI/EcoRI fragment; #12, a 0.3 kb BamHI/PvuII fragment; #38, a 0.5 kb PvuII/XmnI fragment; #19, a 1.1 KpnI/HindIII fragment; #8, a 0.9 kb PvuII/EcoRI fragment; #40, a 0.4 kb EcoRI/PvuII fragment; #9, a 0.5 kb SacI/BamHI fragment; and #10, a 0.7 SacI/EcoRI fragment. Film exposures ranged from 7 to 21 days at −80°C.

Replication Timing Assay

The determination of replication timing by fluorescence in situ hybridization was performed on asynchronous cell populations by the protocol of Trask (1997) and were analyzed as in Kitsberg et al. (1993). Cosmids C3B and KD504 were used as probes for the rhodopsin and *DHFR* loci, respectively. C3B derives from the early-firing rhodopsin origin (Gale et al., 1992), and KD504 is centered in the intergenic region in the *DHFR* locus (C. Ma and J. L. H., unpublished data).

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