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Replication in the Amplified Dihydrofolate Reductase Domain in CHO Cells May Initiate at Two Distinct Sites, One of Which Is a Repetitive Sequence Element

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To study initiation of DNA replication in mammalian chromosomes, we have established a methotrexate-resistant Chinese hamster ovary cell line (CHOC 400) that contains ~1,000 copies of the early replicating dihydrofolate reductase (*DHFR*) domain. We have previously shown that DNA replication in the prevalent 243-kilobase (kb) amplicon type in this cell line initiates somewhere within a 28-kb region located downstream from the *DHFR* gene. In an attempt to localize the origin of replication with more precision, we blocked the progress of replication forks emanating from origins at the beginning of the S phase by the introduction of trioxsalen cross-links at 1- to 5-kb intervals in the parental double-stranded DNA. The small DNA fragments synthesized under these conditions (which should be centered around replication origins) were then used as hybridization probes on digests of cosmids and plasmids from the *DHFR* domain. These studies suggested that in cells synchronized by this regimen, DNA replication initiates at two separate sites within the previously defined 28-kb replication initiation locus, in general agreement with results described in the accompanying paper (T.-H. Leu and J. L. Hamlin, Mol. Cell. Biol. 9:523–531, 1989). One of these sites contains a repeated DNA sequence element that is found at or near many other initiation sites in the genome, since it was also highly enriched in the early replicating DNA isolated from cross-linked CHO cells that contain only two copies of the *DHFR* domain.

Members of our laboratory are studying initiation of replication in the amplified dihydrofolate reductase (*DHFR*) domain in a methotrexate-resistant CHO cell line (CHOC 400) (12). By examining the labeling pattern of amplified restriction fragments on gels after radiolabeling cells at the beginning of the S phase, we previously showed that replication in this 243-kilobase (kb) domain initiates somewhere within a 28-kb region that maps downstream from the *DHFR* gene (4, 5). By using partially single-stranded replication intermediates as probes on cloned fragments from the CHO *DHFR* domain, Burhans et al. (1) have recently presented evidence that DNA synthesis initiates within a single 4.3-kb *Xba*I fragment (XL in Fig. 1B) that is located rather assymetrically within the previously defined 28-kb initiation locus (bracket, Fig. 1B). In an effort to determine the position of replication initiation in the *DHFR* domain with more precision, we utilized an in-gel renaturation technique (14) to increase the resolution of the electrophoretic analysis of radioactive amplified bands from labeled cells. The results of that study (reported in the accompanying paper [8]) suggested that replication actually initiates at two distinct loci that are separated by ~22 kb within the previously defined initiation locus. In the present study, we adopted a totally different approach to analyze initiation in this large domain. We blocked the progress of replication forks emanating from origins at the beginning of the S phase by the introduction of trioxsalen cross-links at 1- to 5-kb intervals in the parental double-stranded DNA template (15). The small fragments synthesized under these conditions (which should be centered around replication origins) were then used as hybridization probes on cloned sequences from the amplified *DHFR* domain. The results of these experiments also suggest the presence of two initiation sites within the amplified *DHFR* domain.

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MATERIALS AND METHODS

Cell culture and synchrony protocol. Cells were cultured and synchronized exactly as described in the accompanying report (8).

Trioxsalen treatment and in vivo labeling protocols. Monolayer cultures in 15-cm dishes (10 per sample) were collected at the G1/S boundary in 30 ml of complete medium containing 10 μg of aphidicolin per ml for 12 h (4). In a darkened room, 15 ml of medium was then removed and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (to 20 mM) and trioxsalen (2.5 μg/ml; Sigma Chemical Co., St. Louis, Mo.) were added. The cells were UV irradiated at room temperature through the medium from above for 2 min at a distance of 10 cm. The lamp (model XX15L; U.V. Products) contained two 15-W tubes with an emission maximum of 365 nm. The cycle was repeated three or five times by successive additions of fresh trioxsalen and 2-min irradiations. After the final treatment, cells were washed in rapid succession with two changes of phosphate-buffered saline and were labeled for 1 h in 15 ml of complete medium containing 20 μCi of [^{3}H]thymidine (60 to 80 mCi/mmol; Dupont, NEN Research Products, Boston, Mass.) per ml. In other experiments, cross-linked cells were labeled for 1 h with 15 μg of bromodeoxyuridine (BUdR) per ml and 5 μCi of [^{3}H]deoxycytidine (30 to 40 mCi/mmol; ICN Pharmaceuticals Inc., Irvine, Calif.) per ml. In the latter protocol, the [^{3}H]deoxycytidine was used only as a tracer for isolation of newly replicated DNA.

Isolation of low-molecular-weight DNA (X-DNA) from

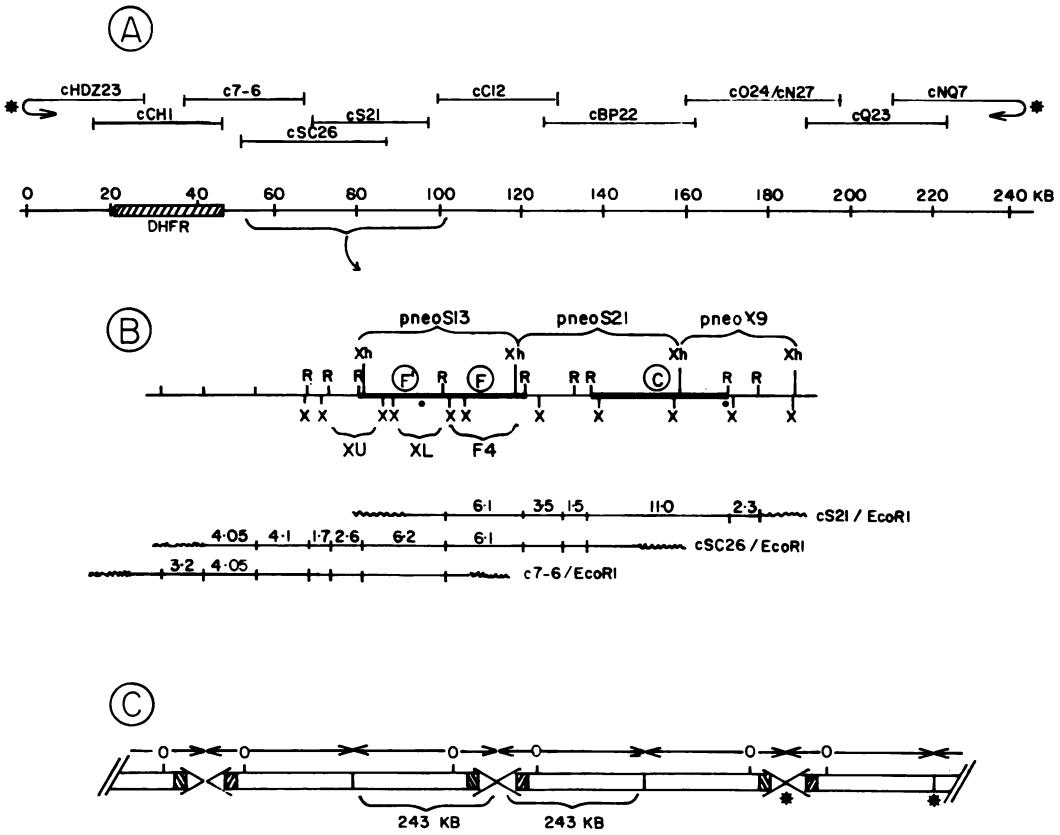

FIG. 1. Sequence arrangement of the amplified *DHFR* domain. (A) Overlapping recombinant cosmids spanning the major 243-kb *DHFR* amplicon. Note that the asterisks at the hairpins in cosmids cHDZ23 and cNQ7 indicate the head-to-head and tail-to-tail interamplicon junctions that define the boundaries of this amplicon. The cross-hatched box on the linear scale represents the *DHFR* gene itself, and the bracket under the scale indicates the initiation region of the amplicon that is expanded in panel B. (B) Restriction map of the initiation locus. The positions of *Eco*RI (R), *Xba*I (X), and *Xho*I (Xh) sites are shown on the upper linear scale, and *Eco*RI ELFs F', F, and C are indicated with a bold line. The clones used in this study are demarcated with brackets (see text). Three overlapping cosmids that together span the initiation locus are diagrammed below, with the sizes of the *Eco*RI fragments indicated. (C) Diagram of the head-to-head and tail-to-tail arrangement of the 243-kb *DHFR* amplicon in the CHOC 400 genome. The cross-hatched boxes indicate the *DHFR* gene itself, and the center of the previously identified 28-kb initiation locus within each repeated unit is marked with an open circle (○). The star below the two arrowheads corresponds to the head-to-head junction in cHDZ23 (also marked with a star in panel A), and the other star corresponds to the tail-to-tail junction in cNQ7 (see star, panel A). The black dots below fragments XL and C in the restriction map in panel B correspond to the two replication initiation sites identified in the accompanying report (8).

cross-linked cultures. After the 1-h labeling period, each 15-cm dish was washed with phosphate-buffered saline and the cells were lysed in 10 ml of 1 M NaCl–10 mM EDTA–50 mM Tris hydrochloride (pH 8) containing 100 μg of proteinase K (Merck & Co., Inc., Rahway, N.J.) per ml and 1% sodium dodecyl sulfate. After 4 h at 37°C, the DNA in each plate (~3×10^7 cells) was flocculated by the addition of 1.5 volumes of ice-cold absolute ethanol and the DNA was spooled on a glass rod and dissolved in 1.5 ml of 10 mM Tris hydrochloride (pH 7.9)–0.1 mM EDTA. The samples from 10 plates were pooled, concentrated NaOH was added to 0.3 M, and the sample was divided into four 3.5-ml aliquots. The four aliquots were applied to 5 to 20% alkaline sucrose gradients (0.3 M NaOH, 1 mM EDTA) and were run at 25,000 rpm for 20 h at 10°C in a Beckman SW28 rotor. The gradients were fractionated and counted, and the average length of the DNA in each fraction was determined by separating a small sample from each on a neutral 1% agarose gel, using a base-denatured 1-kb ladder as the standard. The peak fractions (4 to 6 kb after three cross-linking cycles, and 1 to 2 kb after five cycles) were pooled, neutralized, dialyzed, concentrated to 1 ml, and reapplied to a second 5 to 20% alkaline gradient in the Beckman SW41 rotor, exactly as described in reference 15. After the second gradient, fractions were treated as above, except that the DNA was precipitated with ethanol after the final concentration step. BUdR-labeled DNA was further purified and labeled in vitro as follows. The DNA pellet (usually 10 to 20 μg) was dissolved in 0.2 ml of 0.14 M NaCl–10 mM phosphate (pH 7.2)–0.1% Triton X-100. A 100-μl sample of monoclonal anti-BUdR (24 μg/ml; Becton Dickinson and Co., Paramus, N.J.) was added, and the sample was incubated at room temperature with gentle agitation for 1 h. The antigen-antibody complex was then precipitated by the addition of 12.5 μg of goat anti-mouse immunoglobulin G antibody (1 mg of immunoglobulin G per ml; HyClone Laboratories, Logan, Utah) at room temperature for 1 h, and the suspension was centrifuged in an Eppendorf microcentrifuge at room temperature for 15 min. The pellet was dissolved in 50 μl of 50 mM Tris hydrochloride–10 mM EDTA (pH 8)–0.5% sodium dodecyl sulfate and was digested with 500 μg of proteinase K per ml for 16 h. The DNA was deproteinized twice with an equal volume of chloroform-phenol and once with chloroform and was then precipitated with ethanol. This anti-

BUdR precipitation step achieved an additional 20- to 40-fold purification (based on specific radioactivity). Typically, 80 to 90% of the BUdR-labeled DNA was lost at this step, probably because the ratio of antigen to antibody was not optimal. The resulting pellet (~100 ng) was dissolved in 20 μl of 10 mM Tris hydrochloride–0.1 mM EDTA (pH 8), and 50 ng was labeled with [^{32}P]dCTP by the random primer method (3). DNA concentrations were determined by a fluorimetric assay (6).

Recombinant clones, agarose gel electrophoresis, and transfer and hybridization techniques. The isolation of overlapping cosmids that span the major *DHFR* amplicon type in CHOC 400 cells has been described previously (9). The three *Xho*I fragments from the initiation locus (Fig. 1B) were subcloned from appropriate cosmids into a modified pBR322-based vector (pMMT-neoX [7]) containing the bacterial neomycin resistance gene and eucaryotic transcription signals (the Neo^r marker was used for other experiments not described in this report); an *Xho*I site was added to this vector at the *Sal*I site (Pamela Foreman, unpublished data). *Eco*RI and *Xba*I fragments from the initiation locus were subcloned from appropriate cosmids into pML2 (10) and pEMBL18 (2), respectively. Dot blots and hybridizations were performed exactly as described in Dupont, NEN Research Products brochure no. NEF976, using GeneScreen Plus membranes and a dot-blot apparatus obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). Cosmid or plasmid digests were transferred from agarose gels to GeneScreen Plus by a modification of an alkaline procedure (13); the transfer solution contained 0.4 M NaOH and 0.6 M NaCl. After transfer, the blots were neutralized in 1 M NaCl–0.5 M Tris hydrochloride (pH 7) and were prehybridized for 6 h and hybridized for 24 h at 42°C in 15 ml of the following solution: 1× Denhardt solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin)–50 mM Tris hydrochloride (pH 7)–1 M NaCl–5 mM EDTA–50% formamide–1% sodium dodecyl sulfate. The hybridization solution received a mixture of 1.5 mg of sonicated salmon sperm DNA and either 1 × 10^6 cpm of tritiated X-DNA or 3 × 10^6 cpm of ^{32}P-labeled BUdR X-DNA. In some experiments, the probe additionally received 1.2 mg of blocking CHO genomic DNA that was sonicated to ~500 base pairs (bp) in length. The probes were boiled for 3 min before addition to the bag. After hybridization for 24 h, transfers were washed twice for 10 min with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature, once for 60 min with 2× SSC–1% sodium dodecyl sulfate at 65°C, and once for 60 min with 0.1× SSC at 65°C. All transfers were exposed to X-ray film at −85°C with the aid of an intensifying screen. Dot blots probed with tritium-labeled DNA were sprayed with En^{3}Hance (Dupont, NEN Research Products) prior to film exposure. In most experiments, 2 to 5 weeks of exposure to X-ray film was required for adequate signal detection.

RESULTS

Experimental rationale. We utilized the trioxsalen-UV method of Russev and Vassilev (15) to prepare a DNA fraction that should be enriched in origins of replication for use as a hybridization probe on cloned fragments from the amplicon. In our application of this method, parental DNA strands are cross-linked together at 1- to 5-kb intervals in a cell culture arrested at the G1/S boundary with aphidicolin (i.e., when forks should not have moved significantly away from origins of replication; Fig. 2A and B). When cells are subsequently released from the aphidicolin block and labeled with an appropriate precursor, replication forks should progress only to the first cross-link encountered on either side of the origin (Fig. 2C). Thus, labeling should be confined to a very small zone including and surrounding the origin. The low-molecular-weight DNA synthesized at the beginning of the S phase under these circumstances (hereafter called X-DNA) can then be isolated on alkaline sucrose gradients and used as a hybridization probe on clones from the amplified *DHFR* domain to localize initiation sites with more precision. The problem of background labeling from the thousands of single-copy origins firing at the same time should be largely eliminated, since only the labeled DNA hybridizing to clones from the amplicon should be detected in autoradiograms. Note that the same results would be obtained if low-molecular-weight single-stranded intermediates were isolated from exponentially growing (as opposed to synchronized) CHOC 400 cells, since labeled fragments in this small size range should again be centered around origins. However, the number of origins in the *DHFR* domain that would be firing at a single time in a logarithmic cell population (and, consequently, the amount of *DHFR*-specific X-DNA recovered) would be extremely small and thus too difficult to isolate in sufficient quantities for use as a hybridization probe.

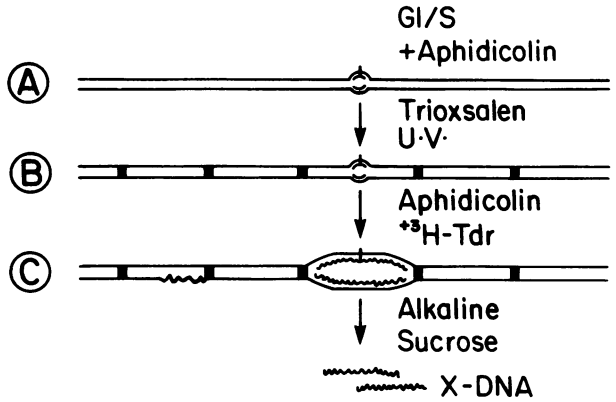

FIG. 2. Strategy for the preparation of an origin-enriched DNA fraction. (A) When cells are collected at the G1/S boundary with aphidicolin after release from a G_0 block, it is assumed that initiation of replication at some origins occurs but that replication forks cannot progress significantly away from these origins because of aphidicolin. (B) Cells are then subjected to the cross-linking procedure, the aphidicolin is removed, and a label such as [^{3}H]thymidine (^{3}H-Tdr) or BUdR is immediately added. (C) In the absence of aphidicolin, the replication forks can progress only to the first cross-links encountered on either side of the origin, with the result that only small fragments centered around the origin should be labeled (indicated by the wavy lines). These can subsequently be isolated as a low-molecular-weight (X-DNA) fraction on an alkaline sucrose gradient. Some repair could also go on at nicks or gaps in the presence of the label (as indicated by the wavy line near the 5′ end of the template, but this labeled DNA would be covalently attached to high-molecular-weight DNA.

Isolation and characterization of X-DNA. When CHOC 400 cells were cross-linked three to five times at the G1/S boundary and were then labeled with 20 μCi of [^{3}H]thymidine per ml during hour 1 of the S phase, labeling was reduced to 0.5 to 2% of the radioactivity incorporated during the same interval of the S phase by control cells that had not been cross-linked (15; B. Anachkova, unpublished observations). When the labeling pattern of genomic DNA was examined by denaturing gel electrophoresis after three or

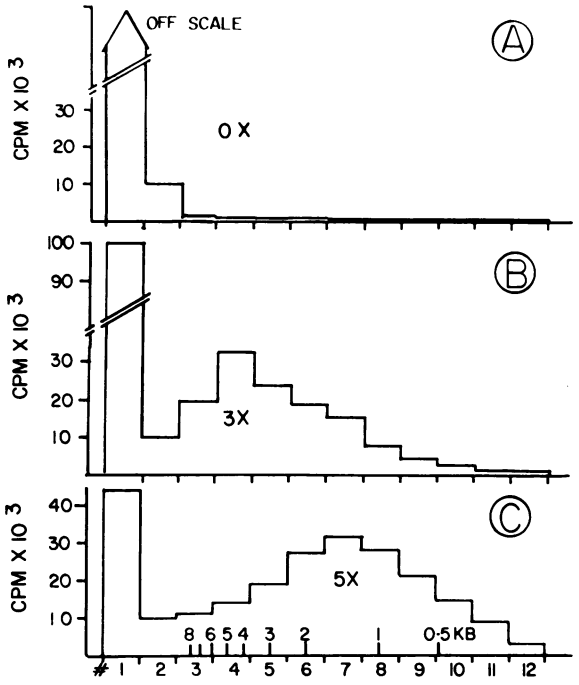

FIG. 3. Size dependence of X-DNA on number of cross-linking cycles. CHOC 400 cells were synchronized at the G1/S boundary and were cross-linked with trioxsalen zero (A), three (B), or five (C) times in the presence of aphidicolin as described in Materials and Methods. The plates were then washed twice with phosphate-buffered saline and labeled for 1 h with 20 μCi of [^{3}H]thymidine per ml. The high-molecular-weight DNA was purified and spooled on a rod as described in the text, and a portion of each sample was electrophoresed on an alkaline agarose gel (prior to centrifugation). The region between the sample well and the dye front was then cut into 12 equal-sized slices, and the radioactivity in each was determined. Fraction numbers are shown at the bottom of panel C, and the positions of a 1-kb ladder relative to the fraction number are shown above.

five cycles of the cross-linking procedure (Fig. 3), at least one-half of the labeled single-stranded material in each case was present in low-molecular-weight fractions. Presumably, the radioactivity in the high-molecular-weight fraction represents small replication intermediates that were not completely released from bulk DNA by the denaturation procedure and/or label that was incorporated into high-molecular-weight DNA by repair processes (Fig. 2C) (15, 16). The mean size of the labeled low-molecular-weight peak after three cross-linking cycles was ~5 kb, and after five cycles, it was ~1.5 kb (Fig. 3B and C).

After two successive sucrose gradients, the specific radioactivity of the low-molecular-weight X-DNA fraction was typically ~2×10^5 cpm/μg, compared with ~4×10^3 cpm/μg for the starting material. Thus, on average, a 50-fold purification of labeled DNA was achieved. However, if this labeled single-stranded DNA was completely pure, the specific activity should be twice that of double-stranded DNA labeled uniformly for one cell cycle with [^{3}H]thymidine of the same specific activity (~10^7 cpm/μg; B. Anachkova, unpublished data). Therefore, only a small percentage of DNA in the low-molecular-weight peak is actually labeled, in agreement with the results of Van der Velden et al. (16). The remainder of the DNA must represent degraded, nonradioactive fragments from the rest of the genome.

Thus, the low-molecular-weight peak from the gradient can be used directly as a hybridization probe when it is labeled intrinsically with [^{3}H]thymidine, but it cannot be labeled in vitro (e.g., with [^{32}P]dCTP) for use as a radioactive probe without further purification. In some experiments, we therefore labeled the DNA with BUdR and a trace of [^{3}H]deoxycytidine and further purified the nascent low-molecular-weight DNA from the second sucrose gradient by precipitation with an antibody to BUdR. This step achieved an additional purification of ~20- to 40-fold, and the resulting DNA preparations typically consisted of 20 to 50% BUdR-labeled DNA. We considered this fraction to be sufficiently enriched in nascent DNA to be labeled in vitro with ^{32}P for use as a probe, since the contaminating nonnascent DNA should be dispersed over the entire genome. The higher energy of ^{32}P relative to ^{3}H reduced the film exposure times required for signal detection. These two different low-molecular-weight preparations are designated X-DNA in the remainder of this report.

Replication in the *DHFR* domain initiates within a region mapping downstream from the *DHFR* gene. The major amplicon type in the CHOC 400 cell line is 243 kb long (Fig. 1A). The multiple copies of this amplicon are arranged in head-to-head and tail-to-tail configurations in the genome, thereby forming giant palindromes (Fig. 1C) (9, 11). Figure 1A shows the arrangement of a series of cosmids that spans this amplicon type. The stars in cHDZ23 and in cNQ7 represent the junctions between amplified units, which correspond to the centers of the palindromes (Fig. 1A and C).

Several cosmids derived from the previously defined initiation locus and flanking regions were immobilized in equimolar amounts on nylon membranes by a dot-blot procedure. The transfers were then hybridized to a purified X-DNA fraction labeled in vivo with [^{3}H]thymidine in hour 1 of the S phase after cross-linking the genomic DNA at 4- to 6-kb intervals (three cross-linking cycles). An equal number of counts from control genomic DNA (labeled uniformly during logarithmic growth) were hybridized to a duplicate filter. The results of this hybridization experiment are shown in Fig. 4.

While the total CHOC 400 genomic control DNA hybridized to approximately the same extent with each of the cosmids (Fig. 4B), the X-DNA fraction hybridized preferentially to the cosmid cSC26 and to a lesser extent to cS21 (Fig. 4A). These two clones contain the previously defined 28-kb initiation locus in its entirety (Fig. 1A and B). Since cSC26 extends to the left of cS21, and since cH1 abuts cSC26 but is not preferentially labeled (Fig. 4A), our results suggest that replication initiates in the region of cSC26 mapping just beyond the 5′ end of cS21 (see map, Fig. 1B). However, some forks must have traveled into sequences represented by cS21, which is labeled about 60% as well as cSC26 (Fig. 4B).

Finer mapping of *DHFR* initiation locus. To increase the resolution of our analysis, we next scanned the entire amplicon by hybridizing transfers of seven different cosmid *Eco*RI digests with BUdR-labeled X-DNA. CHOC 400 cells arrested at the G1/S boundary were cross-linked at 4- to 6-kb intervals (three cycles), and the X-DNA fraction synthesized in the presence of BUdR in hour 1 of the S phase was purified and labeled in vitro with [^{32}P]dCTP. Total CHOC 400 genomic DNA labeled with [^{32}P]dCTP served as a control. In Fig. 5B, it can be seen that the X-DNA probe hybridized significantly only to the 6.2-kb *Eco*RI band in the cosmid c7-6 and to the 6.1- and 11.0-kb bands in cS21 (see map, Fig. 1B). These fragments correspond to the previously identified early labeled fragments (ELFs) F′, F, and C,

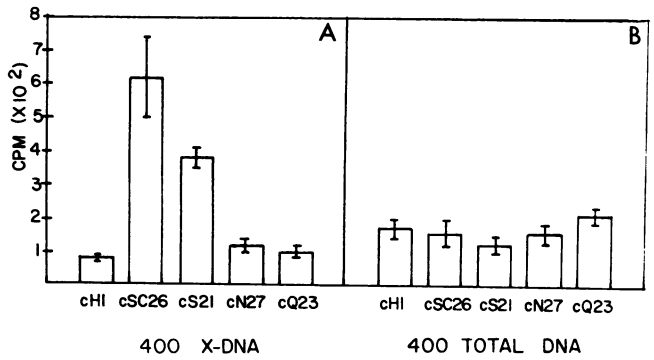

FIG. 4. Replication initiation occurs in a region overlapped by cosmids cSC26 and cS21. CHOC 400 cells were synchronized at the G1/S boundary, cross-linked three times with trioxsalen, and then labeled with 20 μCi of [^{3}H]thymidine per ml for 1 h after removal of aphidicolin. The X-DNA fraction was purified by two successive alkaline sucrose gradients, and 10^6 cpm were hybridized with a dot blot containing triplicate samples of the indicated cosmids (A). A control blot was hybridized with 10^6 cpm of total CHOC 400 genomic DNA that had been labeled uniformly for one cell cycle with [^{3}H]thymidine (B). cS21 (10 μg) was applied in each dot, as well as equimolar amounts of the other cosmids (determined by comparing the respective insert sizes with that of cS21). The averaged results of duplicate experiments are expressed as counts per minute hybridized per 10 kb of insert (after normalizing the cS26 signals between the duplicate experiments). The error bars thus reflect the variation among six dots for each sample.

respectively (4, 5, 8) (note that cosmid c7-6 contains ELF F′ in its entirety but very little of ELF F, while cS21 contains all of ELF F but very little of ELF F′). Thus, this result is in agreement with the dot-blot experiment shown in Fig. 4A. However, the 3.5- and 1.5-kb *Eco*RI fragments that map between ELFs F-F′ and ELF C were not labeled to the extent expected if there is an origin in ELF F′ (as suggested by Burhans et al. [1]), since a replication fork emanating from within ELF F′ would have to travel through the 3.5- and 1.5-kb fragments to reach ELF C, which is also strongly labeled by X-DNA (Fig. 5B). When an X-DNA fraction was prepared from CHOC 400 cells in hour 6 of the S phase, no fragments were preferentially illuminated by the probe (data not shown). This result was expected, since data from the accompanying paper indicated that initiation in the amplified domain is completed by this time (8).

When total CHOC 400 genomic DNA was hybridized to a duplicate filter of the cosmid digests shown in Fig. 5A, almost every band was illuminated to some degree (Fig. 5C), since each restriction fragment from the amplicon is present 1,000 times in the CHOC 400 genome and therefore behaves as a middle repetitive element when used as a probe. However, the signals from several fragments, including ELF F′, were much more intense than others, suggesting that these fragments contain copies of highly repeated sequence elements (Fig. 5C). However, only ELF F′ was preferentially labeled when the X-DNA fraction was used as a probe (Fig. 5B).

It therefore seemed possible that there were two initiation sites in this locus, as we previously suggested (8). The data in Fig. 5 also suggested that the preferential labeling of the

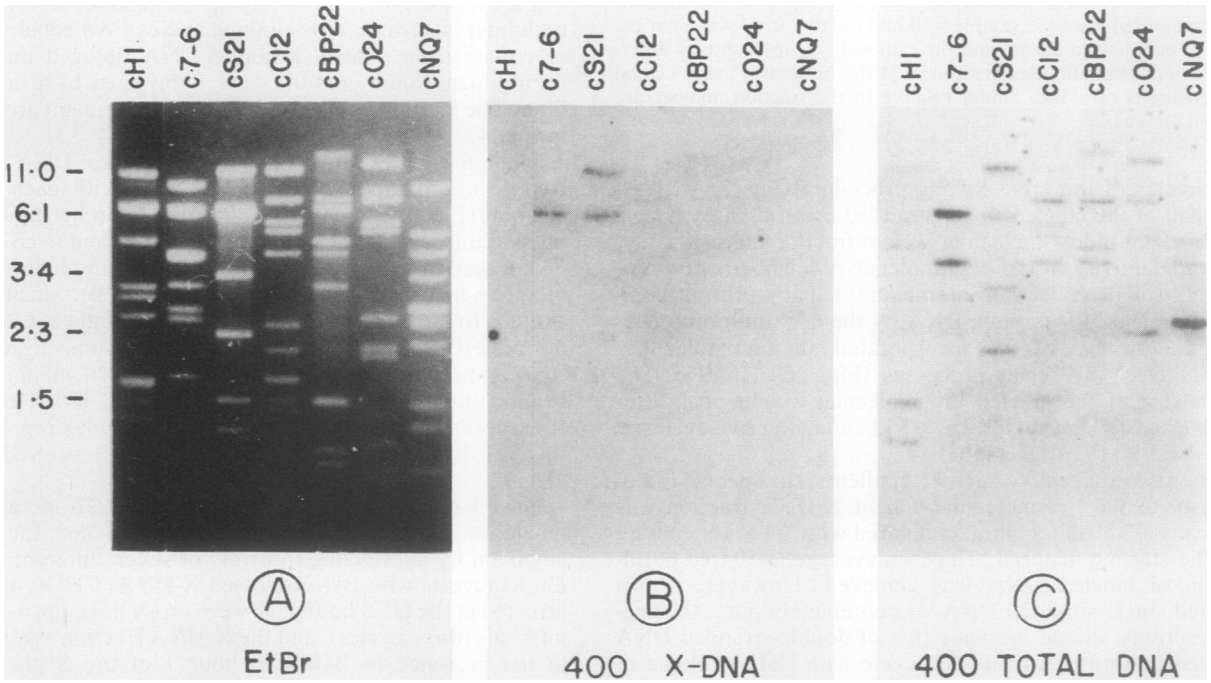

FIG. 5. X-DNA preferentially labels the previously identified early labeled fragments. CHOC 400 cells were synchronized at the G1/S boundary, were cross-linked three times with trioxsalen, and were then labeled with BUdR for 1 h after removal of aphidicolin. The X-DNA fraction was purified by two alkaline sucrose gradients followed by precipitation with anti-BUdR antibodies and was labeled in vitro with [^{32}P]dCTP. Total CHOC 400 genomic DNA was labeled with [^{32}P]dCTP as a control. Approximately 3×10^6 cpm of each of the two samples were hybridized individually to duplicate transfers of the *Eco*RI cosmid digests shown in panel A (EtBr, ethidium bromide). The size markers to the left correspond to the *Eco*RI fragments contained in cS21 (Fig. 1B). (B) Autoradiogram obtained with the CHOC 400 X-DNA probe. (C) Autoradiogram obtained with the CHOC 400 total genomic DNA probe.

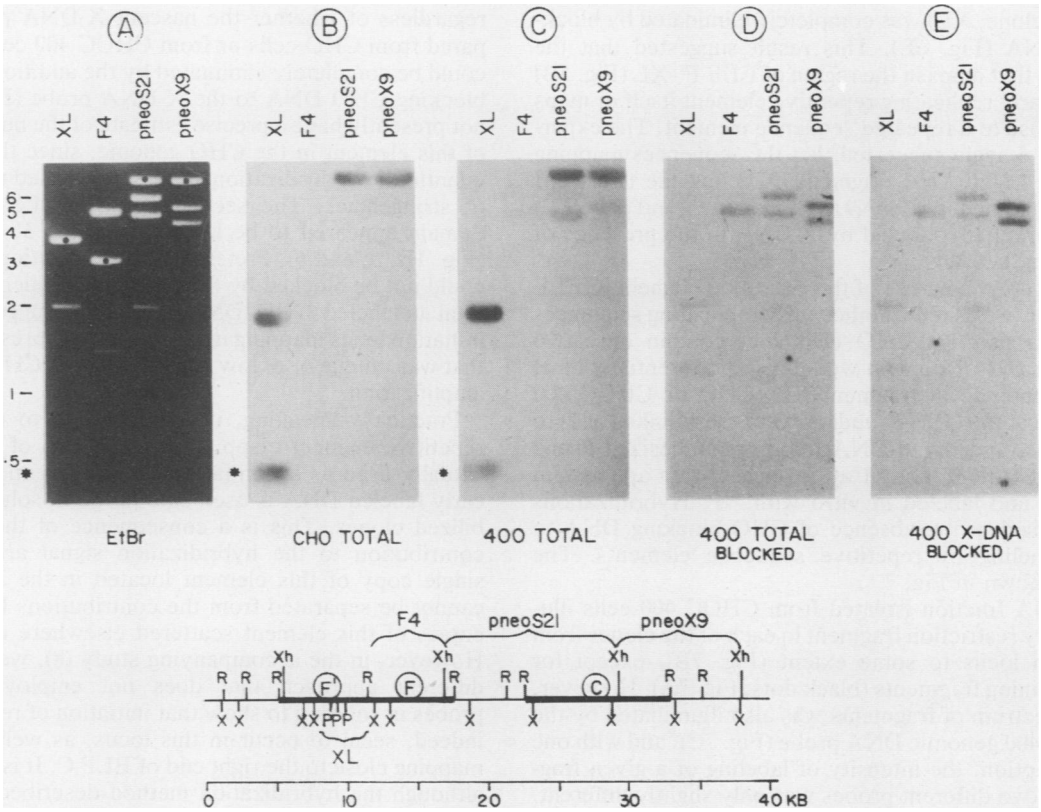

FIG. 6. Preferential labeling of ELF C but not ELF F'/XL by CHOC 400 X-DNA is detected in the presence of blocking CHO DNA. (A) Ethidium bromide (EtBr)-stained gel showing plasmid digests; clone XL was digested with *Xba*I and *Pvu*II, F4 with *Eco*RI and *Xba*I, and pneoS21 and pneoX9 with *Xho*I and *Xba*I. The fragment sizes in the XL digest are 2, 1.2, 0.55, and 0.45 kb, while those in the F4 digest are 4.8, 1.4, and 0.15 kb (see map, Fig. 7). Vector fragments are marked with a black dot, and the relative positions of a 1-kb ladder are indicated. (B and C) The digests shown in panel A were probed with ^{32}P-labeled total CHO (B) or CHOC 400 (C) genomic DNA in the absence of blocking CHO genomic DNA (3×10^6 cpm/15 ml of hybridization fluid in all experiments in Fig. 6). (D) The digests shown in panel A were probed with ^{32}P-labeled total CHOC 400 genomic DNA in the presence of 80 μg of CHO blocking DNA per ml (mixed with probe prior to boiling). (E) X-DNA was prepared and purified from CHOC 400 cells that had been cross-linked five times with trioxsalen and then labeled with BUdR for 1 h after removal of aphidicolin. After being radiolabeled with [^{32}P]dCTP, the X-DNA fraction was mixed with 80 μg of blocking CHO genomic DNA per ml and hybridized to a transfer containing digests of the clones indicated in panel A. A map of the *Eco*RI (R), *Xba*I (X), and *Xho*I (Xh) sites in the initiation locus is shown below, as are the positions of the clones within this locus (note that *Pvu*II [P] sites are shown only for fragment XL). ELFs F', F, and C are indicated by bold lines on the linear map. The stars next to panels A to C indicate the positions of the 450-bp *Pvu*II fragment that is preferentially labeled by CHOC 400 X-DNA in the absence of CHO blocking DNA (see Fig. 7), but not in its presence (panel E).

region represented by ELF F' was due to an early replicating repeated sequence element dispersed throughout the CHOC 400 and CHO genomes, whereas ELFs F and C appeared to be labeled primarily by amplicon sequences in the X-DNA fraction that do not contain significant amounts of highly repeated DNA. This suggestion was further supported by experiments in which we asked whether the preferential labeling of any fragments from the initiation locus by X-DNA from CHOC 400 cells could be blocked by the repeated elements present in total CHO genomic DNA. BUdR-labeled X-DNA (1 to 2 kb long) as well as total genomic DNA from CHOC 400 and CHO cells were each labeled in vitro with ^{32}P and were used to probe digests of subclones from the initiation locus in either the absence or the presence of unlabeled CHO genomic DNA. The results of this experiment and a map of the region are shown in Fig. 6.

When restriction fragments from the initiation locus were probed with total genomic DNA from either CHO or CHOC 400 cells in the absence of any blocking DNA, both a 2-kb *Pvu*II-*Xba*I fragment and a 0.45-kb *Pvu*II fragment from clone XL hybridized strongly (Fig. 6B and C) and therefore must contain repeated sequence elements (there was some hybridization of the CHOC 400 genomic probe to all the fragments, owing to the high copy number of amplicon sequences in the genome). However, no repeated elements were detected in the genomic DNA contained in either pneoS21 or pneoX9. (The two 8-kb fragments from pneoS21 and pneoX9 represent vector, which contains the murine metallothionein promoter and simian virus polyadenylation signals [7], one of which must be repetitive.) All highly repeated sequences from the CHOC 400 probe (Fig. 6D) and the CHO probe (data not shown) were eliminated by 80 μg of blocking CHO DNA per ml.

When CHOC 400 X-DNA was used as a probe in the presence of CHO blocking DNA, only two fragments were preferentially illuminated, both derived from pneoX9 (Fig. 6E). Thus, there appears to be an initiation site near the 3' end of *Eco*RI-C that is composed of sequences that are relatively unique to the amplicon (i.e., single or low copy number prior to amplification). However, the preferential labeling of fragments from the region of ELF F' that was observed in the experiment shown in Fig. 5 (e.g., those in the

4.3-kb *Xba*I clone, XL) was completely eliminated by blocking CHO DNA (Fig. 6E). This result suggested that the initiation site that maps in the region of ELF F'-XL (Fig. 5B) is either an early replicating repetitive element itself or maps extremely close to a repeated sequence element. The experiment in Fig. 6 again suggested that the sequences mapping between the 4.3-kb *Xba*I fragment (XL) and the two *Xba*I fragments contained in pneoX9 (i.e., clones F4 and pneoS21) are not preferentially labeled by X-DNA in the presence of CHO blocking DNA.

To investigate the nature of the repetitive element in ELF F'-XL further, we asked whether early replicating sequences isolated from parental CHO cells that contain only two copies of the *DHFR* domain would also preferentially label sequences mapping in fragment XL. CHO or CHOC 400 cells arrested at the G1/S boundary were cross-linked at 1- to 2-kb intervals, and the X-DNA fraction synthesized in the presence of BUdR in hour 1 after release from aphidicolin was purified and labeled in vitro with ^{32}P. Hybridizations were performed in the absence of CHO blocking DNA to conserve labeling by repetitive sequence elements. The results are shown in Fig. 7.

The X-DNA fraction isolated from CHOC 400 cells illuminated every restriction fragment in each of the clones from the initiation locus to some extent (Fig. 7B), except for vector-containing fragments (black dots, Fig. 7A). However, the same spectrum of fragments was also illuminated by the total CHOC 400 genomic DNA probe (Fig. 7C), and with one notable exception, the intensity of labeling of a given fragment by the two different probes was only slightly different. This results from the fact that this BUdR-labeled X-DNA fraction was contaminated with significant amounts of nonreplicating CHOC 400 DNA that also became radioactive during the in vitro labeling procedure.

The exceptional band is a 450-bp *Pvu*II fragment contained within the 4.3-kb *Xba*I fragment (asterisk, Fig. 7B and C) which was illuminated very strongly by CHOC 400 X-DNA but only weakly by CHOC 400 total DNA. Thus, this 450-bp fragment appears to be markedly overrepresented in the X-DNA fraction isolated from CHOC 400 cells. However, the same 450-bp fragment was also preferentially labeled with X-DNA isolated from parental CHO cells. There are three fragments in this part of the amplicon that contained repeated sequences (Fig. 7E). However, only those that hybridized to the 450-bp *Pvu*II fragment were highly enriched in the X-DNA fraction. Thus, many of the members of this repetitive sequence family must be replicated at the beginning of the S phase.

DISCUSSION

In the present study, we utilized a cross-linking strategy devised by Russev and Vassilev (15) to selectively label small (1- to 5-kb) DNA fragments that are enriched in origins of replication. These fragments were then used to probe cosmids and plasmids from the 243-kb *DHFR* amplicon in CHOC 400 cells to localize replication initiation sites within this large domain. Our results suggest that DNA synthesis initiates at two separate loci that are spaced ~20 kb apart within the previously defined 28-kb initiation locus in the *DHFR* domain (4, 5). These data are therefore in general agreement with those of the previous study (8). One of these sites maps in or very near to a repetitive sequence element that is largely contained within a 450-bp *Pvu*II subfragment of ELF F' (Fig. 1B). This conclusion follows from the fact that the same *Pvu*II fragment was preferentially labeled regardless of whether the nascent X-DNA probe was prepared from CHO cells or from CHOC 400 cells (Fig. 7) and could be completely eliminated by the addition of unlabeled, blocking CHO DNA to the X-DNA probe (Fig. 6E).We do not presently have a precise estimate of the number of copies of this element in the CHO genome, since this will require quantitative hybridization studies performed under a variety of stringencies. The second initiation site in the *DHFR* domain appeared to be located near the 3' end of ELF C (Fig. 1B, 5, and 6). Preferential hybridization to this region could not be blocked by the repetitive sequence elements in total unlabeled CHO DNA (Fig. 6E), suggesting that the initiation locus mapping in ELF C must represent a sequence that was unique or of low copy number in CHO cells prior to amplification.

Practically speaking, it is impossible to prove that the repetitive element mapping in the region of ELF F'-XL is actually used as an initiation site by any method in which early labeled DNA is used as a probe in solution on immobilized clones. This is a consequence of the fact that the contribution to the hybridization signal arising from the single copy of this element located in the *DHFR* domain cannot be separated from the contributions by the multiple copies of this element scattered elsewhere in the genome. However, in the accompanying study (8), we used a totally different approach that does not employ hybridization probes in solution to show that initiation of replication does, indeed, seem to occur in this locus, as well as at the site mapping close to the right end of ELF C. It is important that although the hybridization method described in this report leads to some ambiguities in interpretation because of repetitive elements, it has allowed us to detect the repetitive nature of the initiation site mapping in the region of ELF F'.

Our results agree in part with the data of Burhans et al. (1), who have suggested that there is a single origin of replication in the amplified *DHFR* domain in CHOC 400 cells that maps within the 4.3-kb *Xba*I fragment (fragment X-C in their study, XL in Fig. 1B). This fragment contains the 450-bp *Pvu*II fragment that we suggested is an initiation site in the present study. In the experiments of Burhans et al. (1), partially single-stranded replication intermediates were isolated by benzoylated naphthylated DEAE-cellulose chromatography from early labeled cells and were used to probe cosmid digests or dot blots of cloned *Xba*I fragments spanning the previously identified initiation locus (1). However, the experiments of Burhans et al. (1) did not directly address the repetitive nature of the element contained in the 4.3-kb *Xba*I fragment, although they did mention in the discussion of their paper that this fragment contains a repeated sequence element.

Our results diverge from those of Burhans et al. (1) with regard to the second initiation site that we identified in the right half of ELF C (see map, Fig. 6). In the experiments of Burhans et al. (1) in which nascent DNA labeled in vivo was used as a probe, clones representing the region defined by the right end of ELF C were not included in their analysis, and an initiation site in this region would therefore not have been detected under any circumstances (1). When they utilized DNA labeled in permeabilized cells in the first 5 min of the S phase to probe a series of cloned *Xba*I fragments that actually extended into the right end of ELF C, they again observed significant hybridization only to the 4.3-kb *Xba*I fragment (their X-24) that coincides roughly with ELF F' (Fig. 1B). However, we suggest that preferential hybridization to the 4.3-kb *Xba*I fragment by nascent DNA labeled at such an early time in the S phase resulted from the high

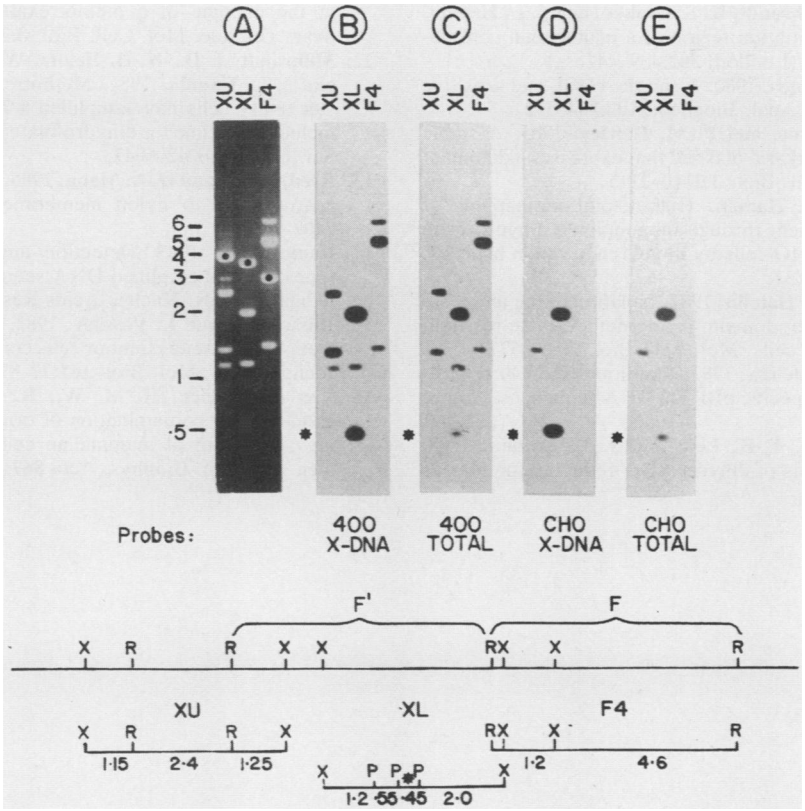

FIG. 7. Preferential labeling of the ELF F'/XL region is due to the presence of an early replicating repeated sequence element. BUdR-labeled X-DNA from CHOC 400 (B) or CHO (D) cells was used to probe digests of the plasmids shown in panel A (black dots denote vector fragments; the positions of the fragments in a 1-kb ladder are indicated). Clone XU was digested with *Xba*I (X) and *Eco*RI (R), XL was digested with *Xba*I and *Pvu*II (P), and F4 was digested with *Eco*RI and *Xba*I. Total genomic DNA from CHOC 400 and CHO cells was labeled in vitro with [^{35}P]dCTP and used to probe replicate transfers (C and E). A map of the relevant part of the initiation locus is shown below (*Pvu*II sites are shown only for clone XL). Note that the stars next to the autoradiograms and in the map indicate the 450-bp *Pvu*II fragment that is preferentially labeled by both CHO and CHOC 400 X-DNA fractions in the absence of blocking CHO DNA. Note that the 2.9- and 6.4-kb bands in the XU and F4 samples, respectively, are partial digestion products.

copy number of this early replicating repetitive element in the genome. This may explain why preferential labeling of the region around the 4.3-kb *Xba*I fragment (e.g., ELF F and ELF F') could not be detected when restriction digests of DNA labeled in the first 5 min of the S phase were separated on agarose gels (i.e., under conditions in which the intrinsic label of the *Eco*RI ELF F' fragment itself could be dissociated from contributions by cross-hybridizing, early replicating, repetitive sequences) (1).

Our finding of two potential origins of replication in this domain is rather surprising, since no other initiation sites were detected in the remaining 220 kb of the *DHFR* amplicon. There are several possible explanations for this result. The first and most straightforward is that there are, indeed, two separate origins in this part of the amplicon that just happen to be closely spaced. It is also formally possible that they fire at slightly different times from one another. A second explanation is that origins of replication in mammalian cells may be more complex than those of simple microorganisms and that both of the initiation sites that we identified in this and the previous study (8) may be part of the same complex origin. The third possible explanation is that there is actually only one origin centered between the two initiation sites that we detected but that artifacts resulting from the use of aphidicolin to synchronize cells at the G1/S boundary prevent labeling of (or result in the eventual loss of) the origin region upon release of cells from the aphidicolin block. To distinguish among these possibilities, it will be necessary to examine other origins of replication and to develop methods for examining the labeling patterns of exponentially growing cells. Both of these approaches are currently under way in our laboratory.

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