A Distinct G_1 Step Required to Specify the Chinese Hamster DHFR Replication Origin

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Nuclei isolated from Chinese hamster ovary (CHO) cells at various times during the G_1 phase of the cell cycle were stimulated to enter S phase by incubation in *Xenopus* egg cytosol. Replication of DNA initiated within the dihydrofolate reductase (DHFR) origin locus in nuclei isolated late in G_1, but at random sites in nuclei isolated early in G_1. A discrete transition point occurred 3 to 4 hours after metaphase. Neither replication licensing nor nuclear assembly was sufficient for origin recognition. Thus, a distinct cell cycle–regulated event in the nucleus restricts the initiation of replication to specific sites downstream of the DHFR gene.

Mammalian origins of DNA replication have been difficult to identify. Although the initiation of replication in cultured fibroblasts is confined to specific, genetically determined chromosomal loci, the size of these loci and the frequency of initiation at particular sequences within these loci remain unclear (*1*–*4*). Furthermore, attempts to identify cis-acting control elements have underscored the apparently limitless number of DNA sequences that have the potential to function as origins (*5*). Extracts of *Xenopus* eggs induce initiation of replication specifically within the DHFR origin locus when intact nuclei (*6*) of CHO cells are introduced as a substrate; however, replication initiates at many more sites with damaged nuclei or bare DNA, demonstrating that origin recognition requires some component of nuclear structure (*7*). In these latter experiments, intact nuclei were isolated from CHO cells that were synchronized exclusively at 4 hours after metaphase (*7*). Given that the well-characterized origins of replication in *Saccharomyces cerevisiae* are occupied throughout the cell cycle by an essential multisubunit origin recognition complex (ORC) (*8*), it was important to determine whether recognition of mammalian replication origins also requires a constitutive element of nuclear structure.

We first examined whether nuclei isolated immediately after formation of the nuclear membrane are already committed to initiate replication at specific sites. CHOC 400 cells (*9*) were synchronized in metaphase by brief (4 hours) treatment with nocodozole followed by selective detachment of mitotic cells (*7*), and were then released into G_1 phase for 60 min—determined to be sufficient time to assemble an intact nuclear envelope in 95 to 100% of cells (Figs. 1 and 3B). Cells were then permeabilized with digitonin (*7*), and intact nuclei ("1-hour nuclei") were introduced into a *Xenopus* egg extract. As a control, nuclei isolated 4 hours after metaphase ("4-hour nuclei") were also introduced into *Xenopus* egg extract. DNA synthesis in both 1-hour and 4-hour nuclei was sensitive to the protein kinase inhibitor 6-dimethylaminopurine (6-DMAP), which specifically blocks initiation of replication (*7*), and began after a similar lag period, indicating that the egg extract mediates assembly of initiation complexes in both types of nuclei at the same rate (Fig. 2A). Because 100% of both types of nuclei were engaged in DNA synthesis (Fig. 2B) at the same overall rate (Fig. 2A), we conclude that similar numbers of replication forks were activated in both 1-hour and 4-hour nuclei.

Initiation of replication at specific genomic loci in 1-hour and 4-hour nuclei was evaluated (Fig. 2, C and D) by the early labeled-fragment hybridization (ELFH) assay (*7*). Nuclei were incubated in *Xenopus* egg extract for 2 hours in the presence of aphidicolin, a specific inhibitor of replicative polymerases, to allow accumulation of newly formed replication forks arrested close to their sites of initiation (*7*). The nuclei were then washed free of aphidicolin, nascent DNA chains were labeled briefly with [α-^{32}P]deoxyadenosine 5′-triphosphate, and the resulting [^{32}P]DNA chains were tested for hybridization with 15 unique probes distributed over a 130-kb region that included the DHFR origin of bidirectional replication (OBR) (*7*). Most of the [^{32}P]DNA synthesized in 4-hour nuclei originated from within the initiation locus (Fig. 2C). In contrast, nascent DNA synthesized near the origins of 1-hour nuclei hybridized almost equally well to each of the probes. In fact, the pattern of early DNA synthesis in 1-hour nuclei was virtually identical to that obtained when replication intermediates were labeled in nuclei from exponentially growing CHOC 400 cells (which serve as a pool of replication forks distributed randomly throughout the DHFR locus) and then tested for hybridization with these same probes (Fig. 2C). To reveal the initiation sites in this region more accurately, we normalized the relative hybridization of early replication intermediates to the 15 probes to that of exponentially growing cells (Fig. 2D). The bell-shaped curve apparent with 4-hour nuclei is consistent with previous origin mapping data at this locus, one possible interpretation for which is a primary region (or regions) of initiation activity localized to within several kilobases, surrounded by secondary sites that constitute a larger initiation zone (*2*, *4*, *7*, *10*). In contrast, no pref-

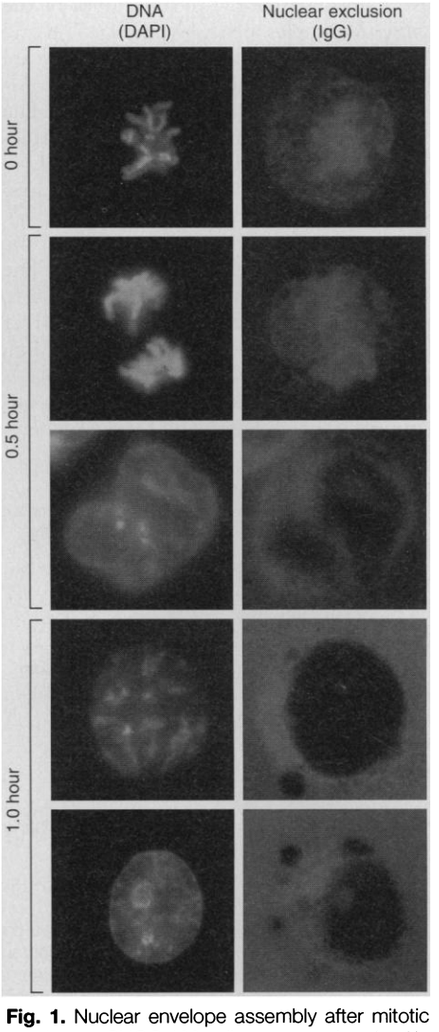

Fig. 1. Nuclear envelope assembly after mitotic synchrony. CHOC 400 cells were synchronized in metaphase as described (*7*) and then released into G_1 for the indicated times, permeabilized with digitonin (*7*), and incubated with 4,6-diamidino-2-phenylindole [DNA (DAPI)] and Texas red–labeled immunoglobulin G [nuclear exclusion (IgG)] (*14*). At 0.5 hour, a mixture of cells in anaphase (top 0.5-hour panel) and telophase (bottom 0.5-hour panel) is observed. By 1 hour, nuclear membranes have completely surrounded chromatin in 95 to 100% of cells, with some nuclei still containing partially condensed chromatin (top 1-hour panel). By 1.5 hours, all cells appear as in the bottom 1-hour panel. Magnification, ×1000.

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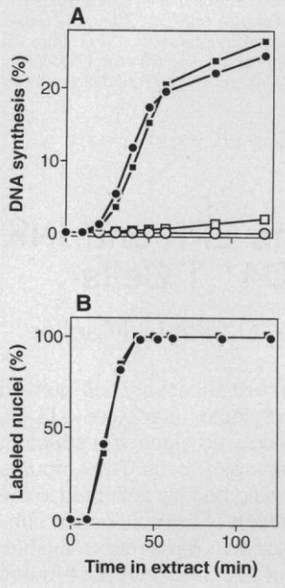
erence for initiation at specific sites was observed with 1-hour nuclei (Fig. 2D).

DNA synthesis stimulated by *Xenopus* egg extract results from de novo initiation of DNA replication and not from DNA repair or the elongation of preprimed replication forks (*7*). Furthermore, site-specific initiation in 4-hour nuclei exposed to *Xenopus* egg extract is apparent in the absence of aphidicolin, providing that replication forks are labeled early in the replication reaction (*7*). To confirm that the difference between 1-hour and 4-hour nuclei was not the result of an artifact imposed by the use of nocodozole, we collected untreated mitotic cells and repeated the experiments shown in Fig. 2, with nearly identical results. Thus, we conclude that *Xenopus* egg extract induced initiation of replication at the same specific sites in 4-hour nuclei as are apparent in cultured CHOC 400 cells, but at sites distributed randomly throughout the DHFR locus in 1-hour nuclei.

To determine the time during G_1 at which CHOC 400 cells restrict initiation to specific regions of the DHFR locus, we prepared nuclei at various times during G_1 and mapped the sites of replication initiation in *Xenopus* egg extract (Fig. 3A). Specificity was not detected until 3 hours after meta-



C

Map position (kb)	Probe	Probe size (bp)	1-hour nuclei Raw data	1-hour nuclei Relative cpm	1-hour nuclei Relative cpm/bp	Exponential nuclei Raw data	Exponential nuclei Relative cpm	Exponential nuclei Relative cpm/bp	4-hour nuclei Raw data	4-hour nuclei Relative cpm	4-hour nuclei Relative cpm/bp
-43	H	1600		7.81	1.96		9.78	2.12		6.19	2.49
-32	I	650		3.01	1.86		2.92	1.56		1.23	1.22
-20	A	643		1.61	1.00		2.21	1.19		1.00	1.00
-5	J	800		2.98	1.49		2.31	1.00		2.93	2.35
12.5	G	685		2.42	1.42		3.33	1.68		12.5	11.8
15.5	B	1532		5.36	1.40		5.59	1.27		28.5	12.0
16.5	C	231		1.18	2.04		1.00	1.50		5.41	15.1
17.5	D	281		1.00	1.42		1.16	1.43		6.70	15.3
18.5	E	380		1.45	1.52		1.91	1.74		9.56	16.2
20	R	887		3.21	1.45		4.20	1.64		22.4	16.2
25.5	F	1250		3.25	1.04		5.44	1.51		32.4	16.7
30	K	700		1.92	1.10		4.37	2.16		18.4	16.9
36	L	1200		3.60	1.20		7.42	2.14		33.8	18.1
81	P	500		1.77	1.42		3.41	2.36		8.52	11.0
86	O	700		3.91	2.24		6.00	2.97		16.6	15.3
	λ	649		0.11			0.20			0.33	

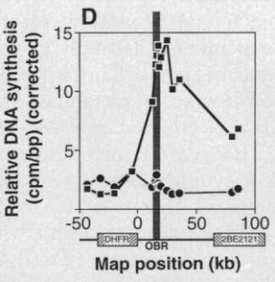
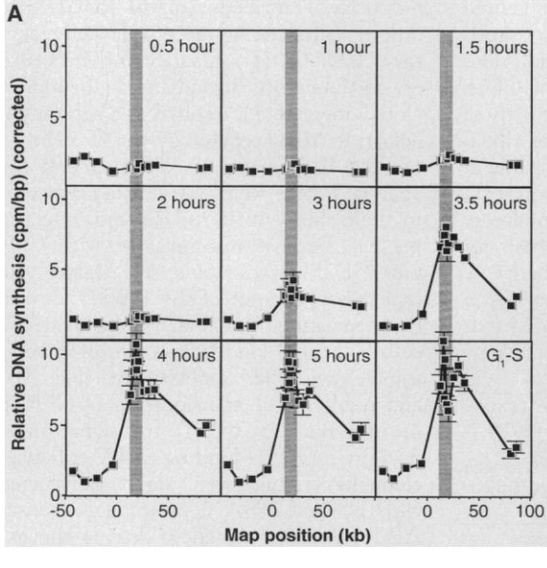


Fig. 2. Uncoupling of replication licensing from origin specification. Nuclei from CHOC 400 cells synchronized at either 1 hour (●) or 4 hours (■) after metaphase were prepared as in Fig. 1 and introduced into *Xenopus* egg extract. (**A**) The percentage of total DNA substrate that replicated in the presence (open symbols) or absence (closed symbols) of 6-DMAP (*15*). (**B**) The percentage of nuclei that were engaged in DNA synthesis (*15*). (**C**) DNA probes were immobilized on slot blots and tested for hybridization with ^{32}P-labeled early replication intermediates from 1-hour and 4-hour nuclei, prepared as described in text. Labeled nascent DNA from exponentially proliferating CHOC 400 cells was also tested for hybridization to the same probes. Probe names and map positions are as described (*7*). Relative counts per minute were obtained with a PhosphorImager (Molecular Dynamics); normalizing to the lowest experimental value; they were also divided by probe size to give relative counts per minute per base pair (cpm/bp). Probe λ is a segment of bacteriophage λ DNA included in each experiment to evaluate the extent of nonspecific hybridization. (**D**) Experimental variation between probes that was not specifically attributable to the synchrony of replication forks close to initiation sites [such as differences in hybridization efficiencies or deoxyadenine content (*7*)] was corrected by dividing the relative value for each probe with synchronized nuclei by the corresponding value for exponentially proliferating nuclei. Because the ELFH assay analyzes relative DNA synthesis after pulse labeling, the differences between probes, not the area under the curve, should be compared. The horizontal axis shows the relative map positions of the probes and includes a diagram to orient the DHFR and 2BE2121 transcription units. The vertical line through the graph shows the position of the previously mapped OBR (*2–4*).



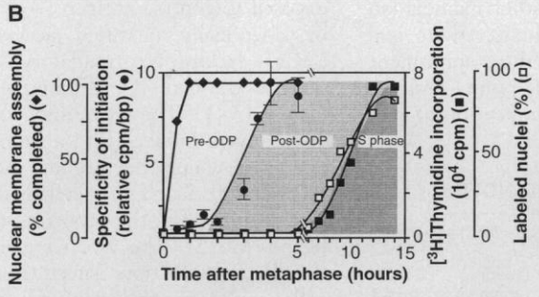


Fig. 3. Timing of the DHFR origin decision point (ODP) during G_1 phase. (**A**) Nuclei were prepared from CHOC 400 cells synchronized at the indicated times after metaphase, and the sites of initiation of replication on incubation in *Xenopus* egg extracts were determined as in Fig. 2D. Data are corrected means ± SEM from at least six independent experiments (SEM only shown when >1). The G_1-S plot shows the mean of three ELFH assays performed after initiation in cultured cells. (**B**) The relative specificity of initiation was defined as the average values for probes B to E [highlighted by the gray line in (A)] in each population of cells and plotted (±SEM) as a function of time after metaphase (●). The completed assembly of a nuclear membrane was monitored after digitonin treatment but before incubation in *Xenopus* egg extract as in Fig. 1 (◆). The onset of S phase was monitored by incorporation of [^{3}H]thymidine into acid-precipitable material during a 30-min labeling period (■) and by the fraction of nuclei that incorporated bromodeoxyuridine in a 30-min labeling period (□) (*7*).

phase and reached a maximum between 3.5 and 4 hours. Because the peak did not increase between 4 and 5 hours after metaphase, and because the height of the ELFH peak after initiation in cultured CHOC 400 cells (G_1-S) was not greater than those of the peaks at 4 and 5 hours, we conclude that the maximum attainable specificity was achieved by 4 hours after metaphase. The relative preference of *Xenopus* egg cytosol for initiation within the DHFR origin locus in nuclei prepared at different times after metaphase is plotted, together with nuclear membrane assembly and the onset of S phase, in Fig. 3B. The first cells entered S phase 7 hours after metaphase and 50% of the cells were engaged in DNA synthesis by 9 to 10 hours after metaphase, consistent with flow cytometric analysis that revealed an average G_1 phase of 10 to 11 hours. These data are also consistent with an increase in the percentage of cells that pass through a discrete regulatory point in G_1 between 3 and 4 hours after metaphase; we have termed this point the origin decision point (ODP) for the DHFR locus.

The data in Fig. 2 demonstrate that the DHFR locus ODP occurs 2 to 3 hours after replication licensing, the mechanism that converts eukaryotic nuclei from a postreplicative (G_2) to a prereplicative (G_1) state (*8*, *11*). Intact mammalian nuclei that have not undergone the licensing step will not replicate in *Xenopus* egg extracts (*11*, *12*). Pre-ODP nuclei have fully assembled nuclear envelopes (Fig. 1) and initiate replication at the same number of sites as post-ODP nuclei (Fig. 2, A and B). The DHFR locus ODP occurs close to the previously identified restriction point (R point), the time after which entry into S phase is independent of growth factors or moderate concentrations of protein synthesis inhibitors (*13*). Thus, it will be of interest to determine whether a similar ODP serves to establish origins of replication at other mammalian chromosomal loci and whether there is a mechanistic link between the commitment to cellular proliferation (R point) and the commitment to initiate replication at specific chromosomal loci (ODP).

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6. Intact nuclei are defined by the exclusion of large fluorescently labeled molecules that lack a nuclear localization signal (Fig. 1). Damaged nuclei are permeable to such molecules.
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9. CHOC 400 is a CHO cell line that contains ~1000 tandemly integrated copies of a 243-kb segment of DNA harboring the DHFR gene, the 2BE2121 gene, and a 55-kb intergenic region that were amplified by stepwise selection in methotrexate (*4*). The pattern of initiation of replication within the amplified locus is indistinguishable from that within the wild-type DHFR locus (*10*).
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14. Cells were permeabilized with digitonin and 2.5 × 10^6 nuclei per milliliter were stained with DAPI (1 μg/ml) and affinity-purified, Texas red–labeled IgG (150 μg/ml) (Jackson Laboratory). Photographs were taken with a Nikon Labophot-2 fitted with a 100× Plan lens (numerical aperture, 1.25) and were composed with Photoshop software.
15. Total DNA synthesis was determined by acid precipitation after incubation in extracts containing [α-^{32}P]deoxyadenosine 5′-triphosphate (25 μCi/ml). 6-DMAP was added to 3 mM. The percentage of labeled nuclei was determined by indirect immunofluorescence after incubation in extracts containing 250 μM bromodeoxyuridine triphosphate. Similar results were obtained in four separate experiments, although the length of the lag period (10 to 30 min) and the total amount of DNA replicated varied with the batch of extract.

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Blocked Signal Transduction to the ERK and JNK Protein Kinases in Anergic CD4$^+$ T Cells

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T cells activated by antigen receptor stimulation in the absence of accessory cell-derived costimulatory signals lose the capacity to synthesize the growth factor interleukin-2 (IL-2), a state called clonal anergy. An analysis of CD3- and CD28-induced signal transduction revealed reduced ERK and JNK enzyme activities in murine anergic T cells. The amounts of ERK and JNK proteins were unchanged, and the kinases could be fully activated in the presence of phorbol 12-myristate 13-acetate. Dephosphorylation of the calcineurin substrate NFATp (preexisting nuclear factor of activated T cells) also remained inducible. These results suggest that a specific block in the activation of ERK and JNK contributes to defective IL-2 production in clonal anergy.

Bretscher and Cohn (*1*) first suggested that antigen-receptor stimulation unaccompanied by critical costimulatory signals could cause the inactivation of mature lymphocytes. Jenkins and Schwartz (*2*) subsequently demonstrated such a functional unresponsiveness in murine CD4$^+$ T cell clones exposed to peptide antigen (Ag) presented by chemically modified accessory cells (ACs) lacking costimulatory molecules such as those of the B7 family of CD28 ligands (*3*, *4*). They determined that these T cells were induced into a state of clonal anergy, in which they become incapable of producing IL-2 and proliferating upon reexposure to Ag and the proper costimulatory ligands (*2*, *5*). The biochemical basis for this IL-2 production defect in anergic T cells remains controversial (*6*); however, a study by Kang *et al.* (*7*) has indicated that IL-2 synthesis is blocked at the level of gene transcription because of poor AP-1 complex assembly and function.

We initiated a study of AP-1 signal transduction using murine CD4$^+$ helper T cell clones (*8*–*10*) in an attempt to identify the biochemical basis for this defect. Overnight treatment of T cells with immobilized monoclonal antibody (mAb) to CD3, in order to simulate Ag-receptor [T cell receptor (TCR)–CD3] occupancy in the absence of costimulatory ligands and to induce clonal anergy (*11*), resulted in a substantial defect in the capacity of the T cells to produce IL-2 (Fig. 1A). Nevertheless, the anergic T cells were not globally deficient in their ability to transduce signals to the nucleus, because restimulation with CD3 plus CD28 mAbs could still induce the rapid disappearance of the 120-kD form of the preexisting nuclear factor of activated T cells (NFATp) (*12*) from a Triton X-100–soluble cytosolic compartment (Fig. 1B) and result in the translocation of NFATp into the nucleus (*13*). To test signal transduction to AP-1–binding DNA enhancer elements, T cells were stably transfected with plasmid DNA containing consensus AP-1 enhancer sequences driving the ex-

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