
Analysis of chromosomal replicons in early embryos of *Drosophila melanogaster* by two-dimensional gel electrophoresis

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ABSTRACT

Chromosomal DNA replication units in early embryos of *D. melanogaster* were studied using two-dimensional gel replicon mapping techniques. DNA was prepared from nuclei encapsulated into agarose beads. This method substantially improved preservation of replication intermediates more than standard DNA preparation methods, and allowed us to detect replication intermediates for even single-copy chromosomal regions without their selective enrichment. Analysis with tandem repeats of histone genes indicated that DNA replication initiates at multiple locations on the repeating unit. The initiation sites were not localized to a defined site, but rather distributed throughout the repeating unit. DNA replication on a single-copy chromosomal region was also suggested to initiate at numerous sites, probably with little regard for the specific DNA sequences.

INTRODUCTION

Recent progress in studies of DNA replication in prokaryotes and eukaryotic viruses have revealed basic features in initiation of DNA replication (for review, see ref. 1, 2). DNA replication usually proceeds bidirectionally from sites known as origins of replication. A replicon is defined as the DNA replicated from a single origin. Most prokaryotic and viral chromosomes consist of a single replicon. Initiation of DNA replication from origins is positively regulated by initiator proteins, such as DnaA protein for *E. coli* and large T antigen for SV40 virus chromosome. These initiator proteins bind to specific nucleotide sequences in the replication origins. The binding of the initiator proteins causes structural distortions of the DNA structure, which lead to the unwinding of nearby DNA regions. Formation of such an open structure is thought to facilitate entry of the complex replication machinery.

In contrast to these prokaryotic and viral chromosomes, eukaryotic chromosomal DNA contains multiple replicons. Organization of the replicons on the chromosomes (size of replicons, timing of their replication in S phase) varies during development in higher eukaryotes. Thus, the essential questions for replication of eukaryotic chromosomes are whether there are

specific origins of replication on chromosomal DNA and whether initiation of replication is triggered by sequence specific binding of initiator proteins as is the case in prokaryotic and viral replicons.

Various attempts have been made to identify replication origins in eukaryotic chromosomes, and these studies have led to apparently conflicting conclusions (for review, see ref. 3, 4). Certain sequence elements (ARS) derived from yeast (*Saccharomyces cerevisiae*) chromosomal DNA allow plasmids containing these elements to replicate autonomously in yeast cells. Recent studies showed that certain ARS elements function as origins on the chromosome. Extensive studies on the DNA sequence requirements for ARS function have revealed an 11bp core consensus sequence essential for ARS function. Similar autonomously replicating sequence elements in mammalian cells have been reported, although their sequence requirements and function on the chromosome are not well understood.

Opposite to the results showing preferred sequences for replication origins, DNA molecules injected into *Xenopus laevis* eggs were found to replicate without sequence preference (5). However, this does not necessarily mean that the replication of such molecules starts from random sites.

To clarify the characteristics of eukaryotic replicons, it is a prerequisite to first localize the replication origins in the context of the chromosome. Among the numerous approaches in attempts to identify replicons on eukaryotic chromosomes, recently developed two-dimensional (2D) gel electrophoresis replicon mapping techniques (6, 7) provide crucial information on replicons, such as the locations of origins, termination sites and direction of replication fork movement. These 2D gel methods have been applied to analyses of chromosomal replicons in yeast (8, 9, 10, 11), amplified chorion genes in *D. melanogaster* follicle cells (12, 13) and amplified DHFR gene region in CHO cells (14, 15). Whereas single-copy loci on yeast chromosomes can be analyzed by these methods, application to chromosomes of higher eukaryotes has been limited to the naturally or artificially amplified chromosomal region. We have used the 2D gel technique to analyze replicons on the chromosome of *D. melanogaster* in early embryos. Detection of replication intermediates for the histone gene repeats (110 copies per haploid) and even for single-copy loci has been achieved.

MATERIALS AND METHODS

Enzymes and chemicals

Restriction enzymes were purchased from Toyobo (Osaka, Japan). Low melting point agarose (SeaPlaque) was obtained from FMC, and agarase from Calbiochem or Sigma. [α - 32 P]dATP was from Amersham. Other chemicals were of reagent grade.

Buffers

Buffer A consisted of 50 mM KCl, 0.5 mM EDTA, 0.05 mM spermine, 0.125 mM spermidine, 0.5% thioglycol, and 5 mM Tris-HCl (pH 7.5), TE buffer 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA, TBE buffer 89 mM Tris-borate (pH 8.0) and 1 mM EDTA, and TAE buffer 40 mM Tris-acetate (pH 8.0) and 1 mM EDTA. SSC consisted of 0.15 M NaCl and 15 mM sodium citrate.

Egg collection

Wild type *D. melanogaster* (Oregon R) was used and eggs were collected essentially as described by Elgin and Miller (16). Flies were maintained at 25°C. Eggs were collected for 1 hr, and further incubated for 1 hr at 25°C. These 1–2 hr eggs involved embryos in the stage from cycle 8 to cycle 12 as described by Foe and Alberts (17). The collected embryos were thoroughly washed with tap water, and freed from their chorions using 5% sodium perchlorite. After extensive rinsing with deionized water and 0.7% NaCl and 0.1% Triton X-100, the eggs were frozen in liquid nitrogen and stored at –75°C until use.

Isolation of nuclei and encapsulation of nuclei into agarose beads

Embryos were suspended in 50 mM Tris-HCl (pH 7.6), 25 mM KCl, 5 mM magnesium acetate, 1 mM EDTA, 0.35 M sucrose, and homogenized by a Potter-type Teflon glass homogenizer at 4°C. The homogenates were filtered through two layers of Miracloth (Calbiochem), and nuclei were collected by centrifugation at 2,500 rpm in a Sorval centrifuge for 10 min.

Crude nuclei were encapsulated into agarose beads according to the modified version (18) of the method of Jackson and Cook (19). Nuclei isolated from 5 g of embryos were washed with 5 ml of buffer A + 0.1% digitonin and resuspended in 1 ml of buffer A + 0.1% digitonin. The nuclei suspension was equilibrated to 37°C and mixed with an equal volume of molten 1% low melting point agarose in buffer A and 4 volumes of liquid paraffin at 37°C in a 50 ml Erlenmeyer flask. The mixture was vigorously shaken by hand for 30 sec. The emulsion was poured into 20 ml of buffer A cooled on ice and the mixture was stirred for several minutes. Agarose beads were collected by low speed centrifugation. Most agarose beads precipitated to the bottom of the tubes, and some floated just beneath the liquid paraffin layer. After removing most liquid paraffin, the contents were mixed thoroughly and centrifuged again. After removing the supernatant by aspiration, the suspension of agarose beads was transferred to a small tube and packed by centrifugation.

Preparation of DNA in agarose beads

The packed agarose beads containing nuclei were suspended in 2 volumes of 1% SDS, 25 mM EDTA. The suspension was shaken for 10 min at room temperature. After centrifugation, the packed beads were resuspended in 2 volumes of 1% Sarkosyl and 25 mM EDTA, and proteinase K (Boehringer Mannheim) was added to 500 μ g/ml. After incubation for 60 min at 37°C,

the agarose beads were washed three times with 2 volumes of TE + 0.1 mM PMSF, and suspended with an equal volume of TE. DNA contents encapsulated in the agarose beads were quantitated by the following method. Agarose was melted by heating at 68°C and the DNA was sheared by vortex mixing so as to be able to penetrate into agarose gel during electrophoresis. After brief electrophoresis in 0.8% agarose in TAE plus 0.5 μ g/ml EtBr, the DNA was quantitated using λ DNA as a standard. The DNA concentration of the agarose beads suspension was usually 8–10 μ g/ml.

Restriction enzyme digestion and recovery of the DNA

The agarose beads containing appropriate amounts of DNA were washed with 2 volumes of the buffer used for digestion by restriction enzyme, and resuspended with an equal volume of the digestion buffer. The suspension was incubated with restriction enzyme (100 units per 1 μ g DNA) for 60 min at 37°C. To facilitate recovery of the digested DNA in the following phenol extraction, agarase (100 units per 100 μ l packed agarose beads) was included during the incubation with restriction enzymes. The solution was extracted twice with phenol/chloroform/*iso*-amylalcohol (25/24/1). DNA was concentrated using either of the following two ways. In most experiments, the volume of the aqueous phase was reduced to about 20 μ l by several extractions with *sec*-butanol. Otherwise, the aqueous phase was diluted with two volumes of TE, and the DNA was precipitated using *iso*-propanol. The precipitated DNA was washed with 70% ethanol and dissolved in 20 μ l of TE+0.1% SDS.

Two-dimensional gel electrophoresis

Neutral/neutral 2D gel electrophoresis. Neutral/neutral 2D agarose gel electrophoresis was carried out essentially by the method of Brewer and Fangman (6). DNA was loaded onto 0.4% agarose gel. The gel was run for 16 hr at 4°C in TBE buffer at 1.2 V/cm. The lanes containing the DNA of interest were excised and placed at the top of a 1.0% agarose gel. Electrophoresis in the second dimension was performed for 5 to 6 hr at 4°C and 4.2 V/cm in TBE buffer containing 0.3 μ g/ml ethidium bromide.

Neutral/alkaline 2D gel electrophoresis. Neutral/alkaline 2D gel electrophoresis was carried out essentially by the method of Huberman et al. (7). DNA was loaded onto 0.4% agarose, and the gel was run for 16 hr at 4°C in TAE buffer at 1.75 V/cm. The lanes containing the DNA of interest were excised, placed at the top of a 1.5% agarose gel, and equilibrated with 50 mM NaOH and 1 mM EDTA. Electrophoresis in the second dimension was performed for 20 hr at 4°C and 2 V/cm in 50 mM NaOH, 1mM EDTA.

Preparation of probe DNA

A plasmid (pKSL100) containing the L unit of histone gene repeats of *D. melanogaster* Oregon R cloned into the *Eco*RI site of pBR322 was provided by Dr. Kaoru Saigo through Dr. Tohru Marunouchi. The 5kb histone gene insert was recloned into the *Eco*RI site of pUC18 (pUHL100). The probe fragments were obtained from purified pUHL100 DNA by digestion with appropriate restriction enzyme(s), and purified by electrophoresis in low melting point agarose. The fragments were labelled with [α - 32 P]dATP using a random oligonucleotide primed DNA labeling kit (Boehringer Mannheim) to specific activity of $6 \times 10^8 \sim 1 \times 10^9$ dpm/ μ g DNA.

Plasmids carrying DNA fragments covering a 50 kb genomic region of the chromosome II of *D. melanogaster* Canton S were provided by Dr. Y. Sano, and probes were prepared as above.

Manipulation of DNA was carried out according to Sambrook et al. (20).

Southern hybridization

After neutral/neutral 2D electrophoresis, the DNA in the gel was denatured in 1.5 M NaCl, 0.5M NaOH after brief depurination in 0.25 N HCl. The gels were neutralized in 1.5 M NaCl, 1 M Tris-HCl (pH 7.4). After neutral/alkaline 2D electrophoresis, these treatments were omitted. The DNA was transferred to Duralon UV (Stratagene) in $10\times$ SSC by capillary blotting. The membranes were baked for 2 hr at 80°C. Prehybridization and hybridization were performed by the method of Church and Gilbert (21). The membrane was immersed in 1% bovine serum albumin, 0.5 M Na phosphate buffer (pH 7.2), 7% SDS for 5 min at 65°C. Hybridization was done in the same buffer containing radiolabeled probe for 16 hr at 65°C.

After hybridization, filters were washed according to Burhans et al. (22). The filters were washed at room temperature in $2\times$ SSC, 2% SDS for 5 min and in $2\times$ SSC, 2% SDS for 30 min at 68°C. The filters were further washed in $0.2\times$ SSC, 0.2% SDS for 30 min at 68°C, and then washed five times in $0.2\times$ SSC, 0.2% SDS at room temperature for 1 min each.

For rehybridization, radioactive probes were removed from the filters by incubation of the filter twice, each for 15 min, at 65°C with 2 mM EDTA and 0.1% SDS preheated to about 90°C (21).

The filters were exposed to a Fuji imaging plate (23) for analysis with a Bio Image Analyzer BAS2000 (Fuji Photo Film, Tokyo).

RESULTS AND DISCUSSION

Two-dimensional gel replicon mapping techniques

Two types of 2D gel replicon mapping techniques were used. In the neutral/neutral 2D gel electrophoresis developed by Brewer and Fangman (6), replicating molecules are identified by their pattern of migration as schematically depicted in Fig. 1. In the first dimension electrophoresis, DNA digested by a restriction enzyme is fractionated mainly according to size in a low concentration of agarose, and then, in the second dimension, the different forms of replication intermediates are separated by electrophoresis in a higher concentration of agarose. The 'simple Y' represents replication intermediates with a single replication fork which traverses the restriction fragment unidirectionally from right to left or from left to right. The 'bubble' represents molecules having an internal replication origin. When the replication origin is located near the center of the fragment, the bubble arc extends to the position of fully replicated molecules. On the other hand, if the origin is located asymmetrically, the replication intermediates follow the discontinuous trajectory starting as bubble structures and then becoming simple Ys when a replication fork passes one end of the fragment. The 'double Y' represents replication intermediates in which two replication forks migrate inwards from the outside origins. The 'asymmetric double Y' shown by a grayed zone in Fig. 1, represents the double Y form with very asymmetrically located replication forks (15).

An independent neutral/alkaline 2D gel replicon mapping technique was developed by Huberman et al. (7). In the first

dimension, the DNA is separated according to mass as in the neutral/neutral method. The DNA in the gel is then denatured in 50 mM NaOH and electrophoresed in alkaline condition. In consequence, the nascent strands dissociated from the parental strands are displayed on a diagonal line according to their length. Size distribution of the nascent DNA strands detected with various small probes provides information on the direction of fork movement, localization of the replication origin and the termination site (for details, see ref. 7 and 8).

Preparation of DNA from nuclei encapsulated in agarose beads

In the initial stages of this work, we prepared DNA by a standard phenol extraction method or CsCl density gradient centrifugation, and enriched replication intermediates by fractionation with BND-cellulose according to the method of Huberman et al. (7). The following problems were noticed in the experiments using these DNA preparations. (1) Many sheared fragments were included. (2) Discrimination of different forms of replication intermediates in neutral/neutral 2D gel electrophoresis was often difficult due to smearing signals appearing in the area where replication intermediates migrate. (3) Detection of replication intermediates for a single-copy chromosomal region was difficult. (4) Detection of nascent strands in neutral/alkaline gel electrophoresis was unsuccessful.

These problems were largely solved by preparing DNA from nuclei encapsulated into agarose beads as described in the Materials and Methods. It was previously reported that large intact DNA could be prepared from cells encapsulated in agarose beads, and could be manipulated without breakage by shear (18, 19). Thus, application of this technique resulted in great reduction of the amounts of sheared fragments, and improved preservation of the replication intermediates. Additional benefits of manipulating DNA in agarose beads was a remarkable reduction of smearing signals in the area where replication intermediates migrate. These smearing signals might be due to an artificial DNA structure formed during the handling of chromosomal DNA at high concentration.

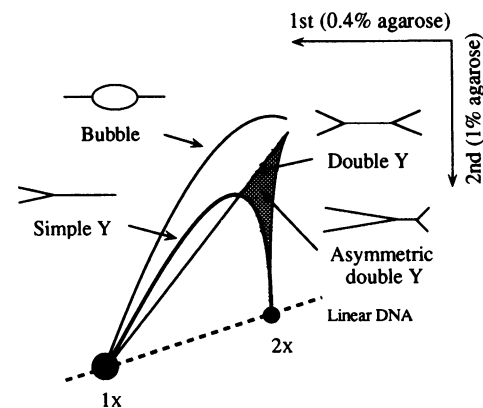


Fig. 1. Schematic diagrams of neutral/neutral 2D gel patterns. Representative patterns of replication intermediates observed in the neutral/neutral 2D gel electrophoresis are shown (for details, see ref. 6). Various types of replication intermediates are separated according to their mass in the first dimension gel, and then separated according to their mass plus shape in the second dimension gel. See text for the meanings of simple Y, bubble and double Y. The grayed area shows the region of double Y forms with very asymmetrically located replication forks (15).

Improvement of the DNA preparation method allowed us to detect replication intermediates using total chromosomal DNA prepared from the early embryos of *D. melanogaster* without selective enrichment for the replication intermediates. Previously, Linskens and Huberman (8) used DNA prepared in agarose beads in replicon analysis of yeast rDNA without recovering DNA from agarose beads prior to electrophoresis. Although they observed that a great reduction of DNA breakage could be achieved by this method, they failed to detect signals of nascent DNA in the neutral/alkaline gel electrophoresis because DNA in agarose beads could not be enriched for replicating DNA. In this work, we recovered DNA from agarose beads by digesting agarose with agarase. Since the recovered DNA can be enriched for replicating DNA by BND-cellulose, this method will be generally applicable to replicon analysis of chromosomal DNA requiring enrichment of replication intermediates.

Neutral/neutral 2D gel analysis of histone gene repeats

Five histone genes (H1, H2a, H2b, H3 and H4) of *D. melanogaster* are represented on a repeating unit of about 5kb long, and each haploid genome contains about 110 copies of the repeating unit (24, 25). Earlier electronmicroscopic analyses of replicating DNA in the early embryos of *D. melanogaster* showed that the mean distance between the adjacent origins was ca. 8 kb (26, 27, 28, 29). Thus, the histone gene repeat units are expected to contain origins of replication active in embryos.

Two types of histone gene repeating units, L (5.0 kb) and S (4.8 kb), are known, but the L unit is three times more abundant than the S unit (24). As we could not discriminate the signals of replication intermediates between the two units, interpretation of the following data will be described for the L unit.

Nuclei isolated from early embryos were encapsulated into agarose beads. DNA prepared as being encapsulated in agarose beads was digested with various restriction enzymes which cut the histone gene repeating unit once (30). The digested DNA was recovered from agarose beads, and was applied to neutral/neutral 2D gel electrophoresis (Fig. 2). Fig. 2A shows the restriction map of the histone gene repeating unit and location of the restriction fragments on the two tandem repeats. Boxes on the fragments represent the middle third of the respective restriction fragments referred to as bubble detection zones by Krysan and Calos (31). It has been suggested that unambiguous bubble arcs can be observed only if replication initiates at the sites within the inner third of a given restriction fragment (11, 31).

Fig. 2B shows the 2D gel patterns obtained using DNA digested by six different restriction enzymes. All six fragments gave essentially the same patterns. The simple Y arcs were predominant. In addition to the simple Y arcs, weaker bubble arcs rising above them and double Y structures with symmetric or asymmetric replication forks were detectable. Presence of the bubble arcs means that at least some histone gene repeats were replicated from origins located within the repeating units. As the bubble arcs are observed irrespective of the sites where the histone gene repeating unit is excised, multiple sites on the repeating unit can serve as origins of replication. All of the bubble arcs seemed to extend to the position of almost fully replicated molecules. It means that replication origins are located near the center of the individual restriction fragments. It is difficult to consider that specific origins are fortuitously located near the center of all fragments excised from the histone gene repeats at different sites. Therefore, the most likely interpretation is that the initiation of

replication in the histone gene repeating unit can occur at numerous sites.

If there is a preferred region to initiate DNA replication, the amounts of the bubble forms are expected to be higher in the restriction fragments whose bubble detection zones include such a region than in the other fragments. Radioactivities in the area of simple Y and bubble arcs were measured by an image analyzer and ratios of bubbles to simple Ys were calculated in two independent experiments (Table 1). Both experiments showed the slight tendency that the bubble/simple Y ratios were higher in the fragments digested by *EcoRI*, *BglII* or *BamHI* and lower in the fragment digested by *XhoI*. However, the differences were too small to conclude that there is a preferred initiation region. These results rather suggest that replication can initiate at various sites on the histone gene repeating unit with similar frequency.

Results shown in Fig. 2B indicate that the simple Y forms are predominant replication intermediates. This suggests that replication in the histone gene repeats initiates only within a subset

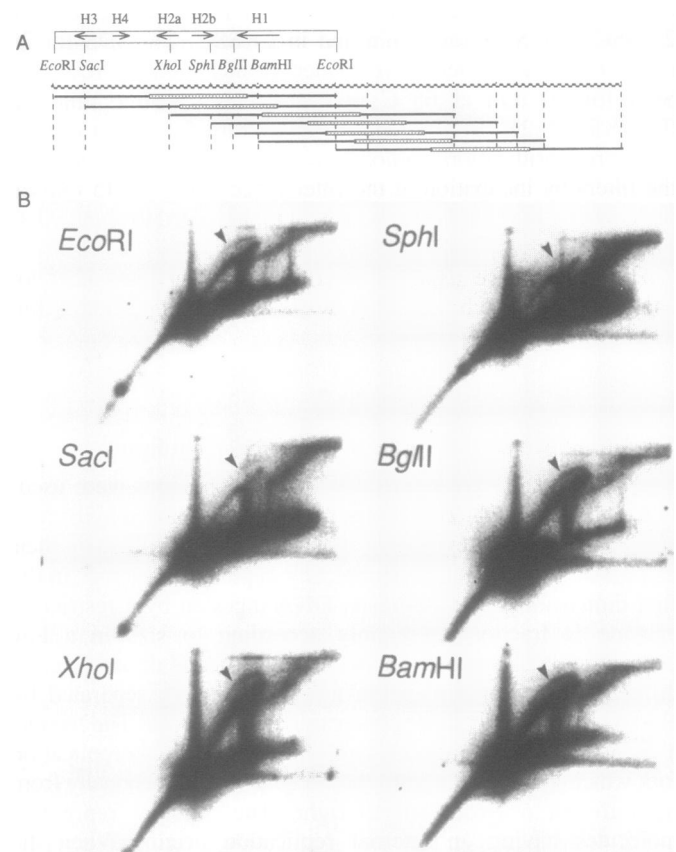


Fig. 2. Analysis of replication intermediates in the histone gene repeats. DNA was prepared from nuclei encapsulated in agarose beads as described in the Materials and Methods. After digestion of the DNA with various restriction enzymes which cut the histone gene repeating unit once, the recovered DNA was concentrated by extraction with *sec*-butanol. Approximately 100 ng of DNA were subjected to the neutral/neutral 2D gel electrophoresis, transferred to Duralon UV, and probed with 5 kb fragment of the histone gene repeating unit. (A) Restriction enzyme cutting sites on the histone gene repeating unit and the restriction fragments excised by digestion with these enzymes. The box on each fragment shows the fragment's bubble detection zone, which corresponds to the inner third of the fragment (31). (B) Neutral/neutral 2D gel patterns of the six restriction fragments. Autoradiograms were taken after 2 hr exposure of the filters to the Fuji imaging plates. Bubble arcs are indicated by arrowheads.

among several repeats. Quantitative estimation of the replication intermediates shown in Table 1 will provide information on the frequency of initiation events. If we assume that replication initiates at random sites in a given restriction fragment, and replication forks proceed bidirectionally with equal rates, we can calculate that about 39% of the replication intermediates are observed as bubble forms. This value would be reduced to about 25%, if the bubble arcs could be detected only when the replication initiates within the bubble detection zone of the fragment. From the results shown in Table 1, the mean of the observed bubble/simple Y ratios is 0.14, giving the value of about 12% for the portion of the bubble forms in the replicating molecules. Thus, initiation of replication in the histone gene repeats is suggested to occur once in two to three tandem repeats, that is 10–15 kb in length.

Neutral/alkaline 2D gel analysis of histone gene repeats

Replication intermediates in the histone gene repeats were further analyzed by another 2D gel technique. DNA digested with *Bam*HI or *Xho*I, which cut the histone gene repeating unit at sites 1.5 kb apart, was subjected to the neutral/alkaline 2D gel electrophoresis. Results with the *Bam*HI digest are shown in Fig. 3. Filters were probed successively with five small fragments shown in Fig. 3A. The autoradiograms are shown in Fig. 3B. The major signals of the nascent strands were detected as diagonal lines from the longest DNA with the length of the parental strand to variable-sized DNAs depending on the probes. Similar results were obtained with the *Xho*I digest. The relationship between the size of the shortest nascent strands detected by each probe and position of the probe in the restriction fragments is shown in Fig. 4 for both the *Bam*HI and *Xho*I digested DNA. In both cases, the shortest nascent fragments of about 600 nucleotides long were detected with the probes from two ends of the respective restriction fragments (probes 5 and 2 in the *Bam*HI digest, and probes 4 and 3 in the *Xho*I digest). On the contrary, central probes (probes 6 and 4 in the *Bam*HI digest, and probe

5 in the *Xho*I digest) detected nascent strands longer than about half the size of the parental strand. These results indicate that the simple Y structures observed as predominant replication intermediates in the neutral/neutral 2D gel method are mixtures of two simple Y structures with replication fork movement in opposite directions.

As the results using the neutral/neutral 2D gel electrophoresis suggest that initiation of replication in the histone gene repeats occurs at nearly random sites, it is expected that a fraction of shorter nascent strands derived from bubble forms can be detected by internal probes. Though not reproduced in Fig. 3, the diagonal lines detected by internal probes seemed to extend further to shorter fragments with much less intensity in the original autoradiograms.

Neutral/neutral 2D gel analysis of a single-copy chromosomal region

Experiments described above showed that replication intermediates from the repetitive histone genes of 110 copies per

Table 1. Quantitative estimation of replication intermediates in the histone gene repeats

Restriction Enzymes	Bubble/simple Y	
	Exp. 1	Exp. 2
<i>Eco</i> RI	0.12	0.19
<i>Sac</i> I	0.10	0.15
<i>Xho</i> I	0.09	0.13
<i>Sph</i> I	nt	0.13
<i>Bgl</i> II	0.13	0.18
<i>Bam</i> HI	0.15	0.17

In Experiment 1, the digested DNA was precipitated with *iso*-propanol. 1.2–1.5 μ g of the digested DNA was subjected to 2D gel analysis. In Experiment 2, the digested DNA was concentrated by extraction with *sec*-butanol. Approximately 100 ng of the digested DNA were subjected to 2D gel analysis. Radioactivities in the simple Y and bubble arcs were quantitated using the Fuji Bio Image Analyzer BAS2000. The ratios are shown. nt, not tested.

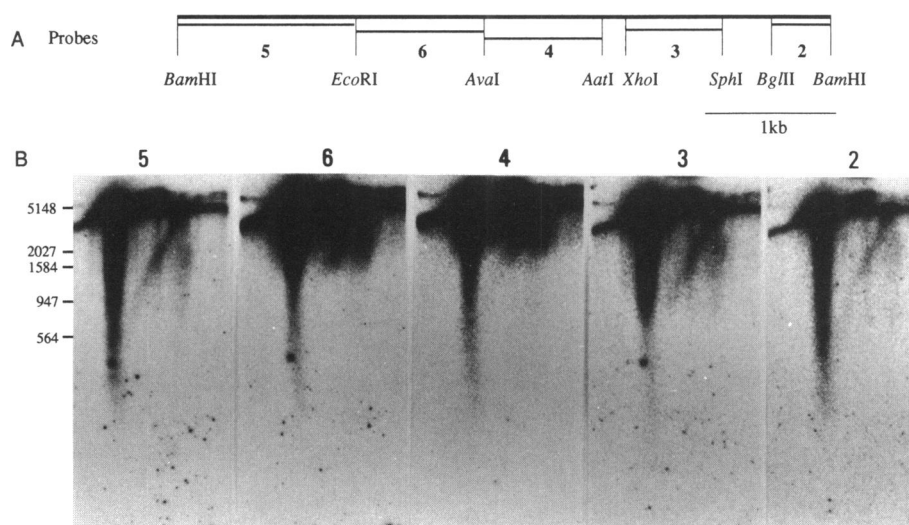


Fig. 3. Neutral/alkaline 2D gel analysis of the histone gene repeats. DNA was digested with *Bam*HI, and concentrated by extraction with *sec*-butanol. Approximately 1.6 μ g of the digested DNA were subjected to neutral/alkaline 2D gel electrophoresis and transferred to Duralon UV. The filters were probed successively with five small fragments in the histone gene repeating unit. (A) Probes used for hybridization. (B) Neutral/alkaline 2D gel patterns with five different probes. Autoradiograms were taken by 2–3 hr exposure to the Fuji imaging plates. Position of size markers (denatured λ DNA digested with *Hind*III + *Eco*RI) are shown in the left. The probe used for hybridization is shown above each autoradiogram.

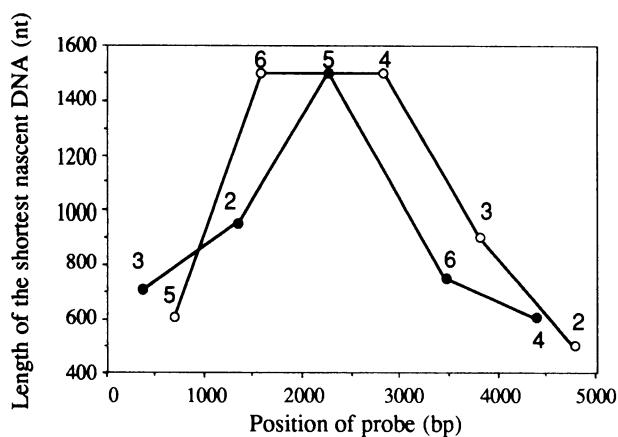


Fig. 4. Relationship between the length of the shortest nascent DNA detected by small probes and their position in the histone gene repeating unit. The *Bam*HI and *Xho*I digested DNA were subjected to the neutral/alkaline 2D gel electrophoresis as described in Fig. 3. The length of the shortest nascent DNA in the predominant diagonal lines detected by each probe was measured. The position of each probe is shown as the distance to the center of the probe from one end of the restriction fragment. The number shown on each symbol represents the probe used. Open circles, *Bam*HI digested DNA. Closed circles, *Xho*I digested DNA.

haploid could be detected with only 0.1 μ g total DNA using the neutral/neutral 2D gel method. This prompted us to analyze replicons of the single-copy chromosomal region.

Eleven restriction fragments ranging from 3.2 kb to 9.0 kb in a 40 kb region of chromosome II were subjected to the neutral/neutral 2D gel analysis (Fig. 5). Fig. 5A shows the restriction map of this region and the restriction fragments used for analysis. The autoradiograms are shown in Fig. 5B. All these fragments gave predominant simple Y arcs as in the histone gene repeats. In addition to the simple Y arcs, we could detect fainter bubble arcs for nine restriction fragments longer than 4 kb. As the electrophoretic conditions were not optimized to be suitable for large fragments (31), the two largest fragments (E8.8 and H9.0) showed somewhat distorted simple Y patterns, and separation of the bubble and simple Y arcs was less clear than the other fragments. Among these restriction fragments, the bubble detection zones of the seven fragments (EB5.2, E5.8 # 2, B4.3, E5.8 # 1, H4.2, H6.4 and HS6.5) did not overlap each other, or overlapped very little. Therefore, this result indicates that there are at least seven replication origins in this chromosomal region.

In conclusion, the most reasonable interpretation of our results is that replication initiates at numerous locations in the histone gene repeats and in the single-copy chromosomal region as well,

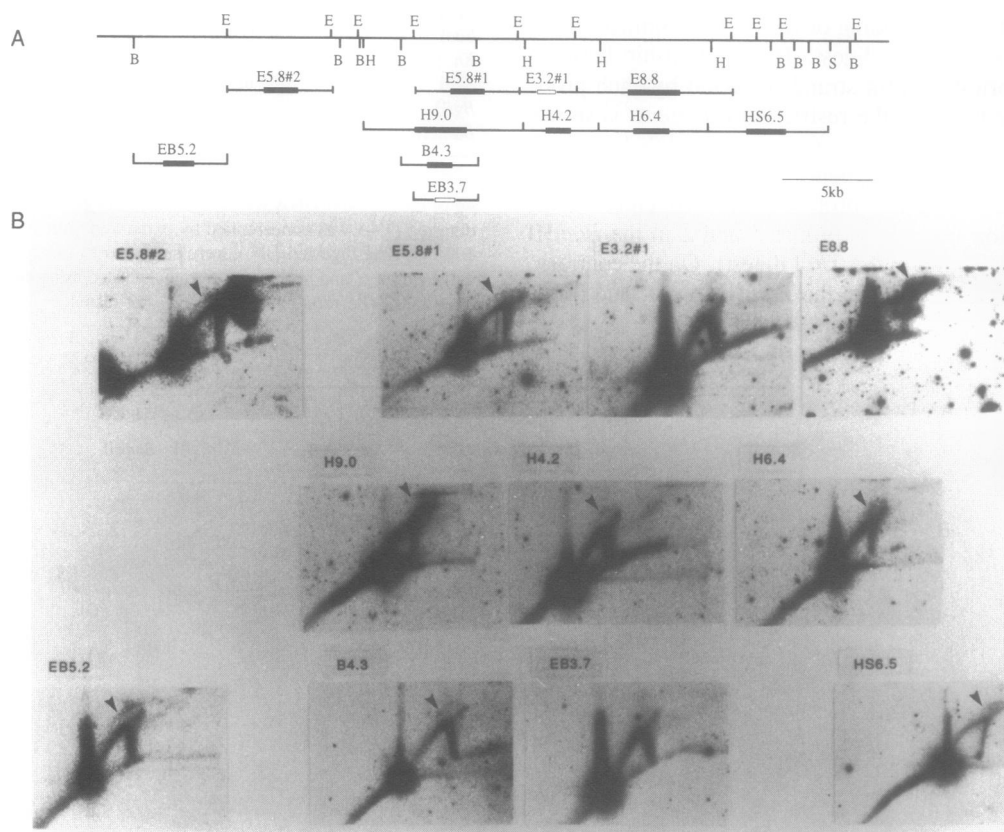


Fig. 5. Analysis of replication intermediates in a single-copy locus. Various restriction fragments covering a 40 kb region of the chromosome II were subjected to neutral/neutral 2D gel analysis. (A) Restriction map and restriction fragments subjected to analysis. The box on each fragment shows the fragment's bubble detection zone, while the black ones represent where the bubble arc was detected for that restriction fragment. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; S, *Sal*I. Sites for *Hind*III and *Sal*I between the two *Bam*HI sites on the left have not been mapped. (B) Neutral/neutral 2D gel patterns. Chromosomal DNA in agarose beads was digested by *Eco*RI, *Hind*III, *Bam*HI, *Eco*RI + *Bam*HI or *Hind*III + *Sal*I. The digested DNA was concentrated by extraction with *sec*-butanol, except the *Eco*RI digest which was precipitated with *iso*-propanol. Two to 4 μ g of the DNA were used. Autoradiograms were taken by exposure of the filters for 2 to 42 hr to the Fuji imaging plates. Bubble arcs are indicated by arrowheads.

probably with little regard for the specific DNA sequences. Such relaxed requirements for specific DNA sequences to replication origins may reflect the specialized nature of rapidly dividing embryos as in the case of *Xenopus* eggs, in which any injected DNA can replicate (5). However, recent results reported by Krysan and Calos (31) strongly suggest that it may be a common feature of the chromosomal replicons in higher eukaryotes. Using the neutral/neutral 2D gel method, they showed that replication initiates at multiple locations on the plasmid carrying the human DNA sequences, in contrast to the Epstein-Barr virus vector which replicates from a fixed origin. Their result indicates an apparent difference between chromosomal replication origins and a virus origin in the same experimental conditions, and is consistent with the replicon analysis in the amplified hamster DHFR gene by the 2D gel method (15). By contrast, conflicting results showing the presence of fixed origins of replication in mammalian cells have also been reported using other techniques (22, 32, 33). Further studies are needed to clarify whether a low degree of sequence specificity in the replication origins is specific to rapidly dividing embryos or is a common feature of chromosomal replicons in higher eukaryotes.

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