SIZE CLASSES OF REPLICATION UNITS IN DNA FROM SEA URCHIN EMBRYOS

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ABSTRACT

Sea urchin DNA containing replication structures was isolated from two to four cell stage and blastula stage embryos, and examined by electron microscopy. In addition to the expected eye forms, we also observed molecules with large internal single-stranded gaps. Such structures were not present in DNA devoid of replicating molecules such as that isolated from sea urchin sperm. When the size of eye forms and interbubble distances between the two stages were compared, there was no detectable difference. In both stages, we observed two distinct size classes of bubbles and of interbubble distances. In the case of bubble sizes, the smaller size class was comprised of clustered microbubbles that ranged from 200 base pairs to 1 Kilobase (kb) with a mean of 432 base pairs. The large eye forms measured 1-35 kb with a mean of 6.8 kb. Interbubble distances also yielded two distinct populations, with the smaller class ranging from 400 base pairs to 2.3 kb (mean = 1.1 kb) and the larger population ranging from 2.8 to 36 kb (mean = 10.9 kb). Although other possibilities cannot be entirely excluded, the data support the contention that a substantial fraction of the larger eye-form population arises from the fusion of the clustered microbubbles.

KEY WORDS replication · electron microscopy · sea urchin embryos

Replication in eukaryotic chromosomes initiates at origins arranged tandemly along the DNA molecule (10). The unit of replication, the replicon (15), has been defined as the distance between adjacent origins. When cultured cells with S phases of 6 h or longer are examined by DNA fiber autoradiography, putative replicons with an average size of ~30 μm are detected (7, 10). One short-coming of the autoradiographic approach, however, is that it cannot resolve replication units smaller than 4 or 5 μm. Electron microscope examination of replicating DNA from early insect embryos with very short S phases (2, 16, 12, 17) has revealed an average replicon size between 2 and 4 μm, which is much smaller than that detected by DNA fiber autoradiography in more slowly dividing cultured adult cells. Callan (4) has proposed that the lengthening of the S phase, such as that which occurs during early embryogenesis, is primarily controlled by a reduction in the frequency and absolute number of replication initiation events. Such a reduction would also result in an effective increase in replicon size. Recently, however, an electron microscope study of replicating DNA from cultured Chinese hamster cells has shown that these cells, which have a 6-h S phase, contain replicons as small as any seen in embryonic systems (3, 14).

Evidence from Drosophila virilis embryos suggests that some replication structures <0.3 μm long may represent a separate class of replication unit (17). In the electron microscope, these units are identified as clusters of replication bubbles. The bubbles in any one cluster are of similar size, suggesting that the units may have been initiated...
synchronously. In DNA from *Drosophila virilus* embryos, the distance between centers of adjacent clusters of microbubbles approximates the center-to-center distance between adjacent bubbles of the larger size class (17), suggesting that the latter may originate as the result of fusion of the microbubbles. A recent study on DNA from embryos of the sea urchin, *Paracentrotus lividus*, revealed only microbubbles and no eye forms of the larger size (1).

In this report, we describe measurements made on replicating DNA molecules from early cleavage and mid-blastula embryos of the sea urchin, *Arbacia punctulata*. We observe bubbles of the large-size class, as well as clustered microbubbles (i.e., \(<1\) Kilobase [kb]), at both developmental stages. Center-to-center distances between adjacent bubbles on the same molecule were similar in the two stages. Likewise, no significant difference in the range or average size of replication bubbles between the two developmental stages was detected.

**MATERIALS AND METHODS**

**Preparation of Fertilized Eggs and Embryos**

Gamete shedding was induced in both sexes of *Arbacia punctulata* by intercoelomic injection of 0.5 M KCl. Freshly laid eggs were washed twice in seawater and fertilized with a 5,000:1 dilution of “dry” sperm. Embryos developed at 22°-24°C in seawater containing 10 mM \(\beta\)-mercaptoethanol to prevent formation of the fertilization membrane (8). At the appropriate developmental stage, embryos were collected by centrifugation, resuspended in lysis solution (100 mM Tris, 10 mM Na\(_2\)-EDTA, 1% Sarkosyl, pH 8.5) and disrupted in a Dounce homogenizer (Kontes Co., Vineland, N. J.). The lysate was digested with Proteinase K (100 \(\mu\)g/ml) overnight at 24°C.

**DNA Isolation**

After two extractions with chloroform:isoamyl alcohol (24:1), the lysate was dialyzed into 10 mM Tris, 1 mM Na\(_2\)-EDTA, pH 8.5. Samples were adjusted to a density of 1.70 g/cm\(^3\) with CsCl and centrifuged to equilibrium at 33,000 rpm for 48 h in a Spinco 50 Ti rotor (Beckman Instruments, Inc., Palo Alto, Calif.). Gradients were monitored for absorbance at 260 nm in a Giffford continuous flow spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio), and collected in 20-drop aliquots. Fractions containing DNA were pooled, and the CsCl was removed by dialysis against 10 mM Tris, 1 mM Na\(_2\)-EDTA, pH 7.5.

**Electron Microscopy**

Samples were prepared for electron microscopy by the protein film technique as modified by Davis et al. (6). DNA was spread from 50% formamide onto a hypophase of water, picked up on carbon-coated grids, and rotary-shadowed with platinum: palladium. Grids were examined on an RCA EMU-3 electron microscope, and molecules containing one or more eye forms were photographed at \(\times 7,500-11,000\). Care was taken to avoid duplicate counting of molecules, and any molecule which could not be followed from end to end was excluded from the sampling. Photographic negatives were projected to trace the molecules, which were then measured on a Tektronix tracing table (Tektronix, Inc., Beaverton, Oreg.) programmed for length determinations. Lengths in computer units are converted to kilobase units by comparison to PM-2 phage DNA (6.4 megadaltons; 9,725 base pairs) spread either together with the urchin DNA or under similar conditions.

**RESULTS**

DNA from early cleavage (two to four cells) and from mid-blastula embryos was isolated and examined by electron microscopy. In addition to high molecular weight, linear, double-stranded DNA, four other populations of molecules were observed. These include: (a) circular molecules which we have identified as mitochondrial DNA on the basis of size (15,175 ± 65 base pairs, SEM), (b) molecules containing single-stranded DNA, four other populations of molecules were observed. These include: (a) circular molecules which we have identified as mitochondrial DNA on the basis of size (15,175 ± 65 base pairs, SEM), (b) molecules containing single-stranded DNA, (c) forked structures, and (d) molecules with eye forms or bubbles (Figs. 1a and 2).

The latter two classes of structures are generally considered to represent molecules undergoing replication, with forked structures presumably arising by breakage of bubbles during isolation of the DNA. To avoid errors involved in reconstructing the original configuration of forked molecules, we did not include them in our sampling. In contrast, the origin of molecules with single-stranded tails or gaps (Fig. 1b and c) is uncertain (9); however, they do appear to be enriched in actively replicating systems. A molecule with two single-stranded internal gaps is presented in Fig. 1b. We find such molecules in DNA from early cleavage and blastula stage embryos but not from sperm or unfertilized eggs, which renders it unlikely that they are strictly an artifact of the isolation procedure. Whether these gaps are functionally involved in the replication process remains unclear. However, we have observed them on molecules containing identifiable replication bubbles (Fig. 1c). The presence of single-stranded gaps or nicks in the DNA of *Strongylocentrotus purpuratus* embryos at various developmental stages has previously been inferred from S1 nu-
cleavage studies (5), and such gaps have also been visualized in DNA isolated from Paracentrotus lividus embryos (1).

Molecules containing eye forms comprised <1% of the total number of molecules inspected. As predicted for replicative structures, the two arms of the bubbles were of equal length and, for cases in which short single-stranded regions could be distinguished at both forks, the single strands were always arranged in trans-configuration (11). The molecules could be divided into two classes based on the size of the eyes (Fig. 3). Fourteen of
FIGURE 2 A DNA molecule with a cluster of microbubbles. The bar represents 0.5 kb.

FIGURE 3 Histogram of bubble sizes pooled from early cleavage and blastula stages depicting two size classes of eye forms, $n = 275$.

The 138 molecules measured contained bubbles <1,000 base pairs long (mean = 440 base pairs) that were generally clustered on the molecules. The remaining eye forms ranged in length from 1,000 to 35,000 base pairs (mean = 6,800 base pairs). These data (Fig. 3) are pooled from early cleavage and from blastula stages since measurements of bubble size from each of these developmental stages revealed no detectable differences.

A most interesting observation to arise from these data is that the eye forms fall into two discrete size classes. The size distribution described in Fig. 3 is presented in logarithmic form, since measurements extend over two orders of magnitude. The data clearly do not conform to a single log normal distribution, but are instead comprised of two distinct populations of bubble sizes. This interpretation is borne out by presenting the data as a probability plot, which again describes two distinct components (Fig. 4).

A similar distribution was observed with the interbubble distances. Measurements of apparent replicon length were made on molecules such as the ones shown in Fig. 1a and in Fig. 2 by determining the distance between the centers of adjacent bubbles. Again, no significant differences were found either in the distribution or in the average length of interbubble distance between cleavage and blastula embryos (data not shown). Measurements pooled from both stages revealed two distinct but overlapping distributions of the center-to-center distances between adjacent bubbles (Fig. 5). The larger population has a mean of
FIGURE 4 The distribution of bubble sizes presented as a probability plot.

FIGURE 5 Histogram of center-to-center distances measured between adjacent replication bubbles, \( n = 132 \).

\( \sim 13.4 \) kb. The smaller population with a mean center-to-center distance of \( 1.1 \) kb arises from the small eye forms which tend to be clustered along the molecules (Figs. 2 and 3).

DISCUSSION
The data describe two discrete size classes of eye forms in DNA isolated from two developmental stages of sea urchin embryos. The fact that they are detected only in replicating systems and not in DNA from unfertilized eggs or sperm treated identically renders it unlikely that they are products of localized denaturation. The smaller population of eye forms is comprised of microbubbles with an average size of \( \sim 450 \) base pairs. Such structures have been previously reported in DNA isolated from embryos of the sea urchin *Paracentrotus lividus* (1). In contrast to this earlier study, which detected only microbubbles, we find a larger size class of bubbles with an average size of \( \sim 7 \) kb. When treated with a single-strand-specific nuclease, the microbubbles are eliminated (1). This is consistent with our observation that in some instances one of the arms of the microbubbles appears single-stranded, perhaps due to the loss of unligated Okazaki fragments. Although the microbubbles are usually found as clusters, they occasionally occur as isolated structures, and are also sometimes found on the same molecule as bubbles of larger size. Microbubbles have been reported to be unstable, disappearing within a few days upon storage in electron microscope buffer at \( 4^\circ \)C (1); however, we have been able to detect microbubbles for up to 2 mo, after storage in \( 10 \) mM Tris-HCl, \( 1 \) mM Na-EDTA, pH 7.5.

The failure of Baldari et al. (1) to detect the larger bubbles in another sea urchin species is bothersome. It is unlikely, however, that the absence of large bubbles can be attributed strictly to technical difficulties, since those authors have observed large bubbles in DNA isolated from other organisms. The differences between our observations and those of Baldari et al. (1) may, in fact, reflect true difference in replication between the two sea urchin species.

The discontinuity in distribution of bubble sizes and of center-to-center distances argues that replication at a given site may frequently initiate not as a single event but as a cluster of events, giving rise to the clusters of microbubbles that we and others have reported (1, 3, 17). Subsequent fusion would give rise to a quantal increase in bubble sizes and would yield a separate population comprised of larger bubble size and interbubble distances. The observation that the size of microbubble clusters falls within the size range of large bubbles supports this argument. Such a precursor-product relationship between clustered microbubbles and large bubbles has also been postulated by Zakian (17) who found that, in *Drosophila viridis* embryos, cluster-to-cluster distances fall into
modes of ~4,000 base pairs which roughly correspond to the center-to-center distances of the larger bubbles in that system. The combined data in Figs. 3 and 5 support this contention and indicate that in Arbacia punctulata two populations of replication units can be distinguished on the basis of size. Although supportive of such a precursor-product relationship, the possibility that a substantial fraction of larger replication units may arise from a single initiation event, and that microbubbles might reflect a series of closely spaced initiation events in clustered repeated sequences such as satellite DNA has not been ruled out.

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