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The replicon revisited: an old model learns new tricks in metazoan chromosomes

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The origins of DNA replication were proposed in the replicon model to be specified genetically by replicator elements that coordinate the initiation of DNA synthesis with gene expression and cell growth. Recent studies have identified DNA sequences in mammalian cells that fulfil the genetic criteria for replicators and are beginning to uncover the sequence requirements for the initiation of DNA replication. Mammalian replicators are composed of non-redundant modules that cooperate to direct initiation to specific chromosomal sites. Conversely, replicators do not show strong sequence similarity, and their ability to initiate replication depends on the chromosomal context and epigenetic factors, as well as their primary sequence. Here, we review the properties of metazoan replicators, and discuss the genetic and epigenetic factors that determine where and when DNA replication is initiated.

Keywords: chromatin domain; DNA replication; mammalian chromosomes; origins; replicators; replicon

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Introduction

DNA replication is a highly orchestrated process that precisely duplicates the genome once during each cell-division cycle. The replicon model, which was formulated more than four decades ago, laid the foundation for our understanding of the control of DNA replication in the cell cycle (Fig 1; Jacob *et al*, 1963). Specific sequence elements, termed replicators, were postulated to genetically determine replication-initiation sites on DNA molecules. The interaction of replicators with *trans*-acting regulatory factors, called initiators, was proposed to activate initiation in response to specific signals from the cell-cycle machinery. Replicators were readily identified in prokaryotes, DNA viruses and lower eukaryotes by their ability to mediate the extrachromosomal replication of plasmids, and initiators were identified on the basis of their ability to bind to replicators. These findings validated the model and revealed the basic mechanisms by which eukaryotic cells regulate DNA replication (Bell & Dutta, 2002).

In mammalian chromosomes, DNA replication begins at multiple initiation regions (IRs) or origins, with an average spacing of 100 kb (Huberman & Riggs, 1968). About two-dozen metazoan IRs have been physically or biochemically mapped so far (Gilbert, 2001; Bell, 2002; Gerbi *et al*, 2003). However, until recently, genetic evidence for metazoan replicators that specify initiation sites has been elusive. A handful of metazoan replicators, which are defined by their ability to trigger the initiation of DNA replication *in cis* when placed at ectopic chromosomal locations, have now been identified (Fig 2). Below, we review the properties of these replicators and discuss the roles of *trans*-acting factors and chromatin structure in regulating where and when replication is initiated.

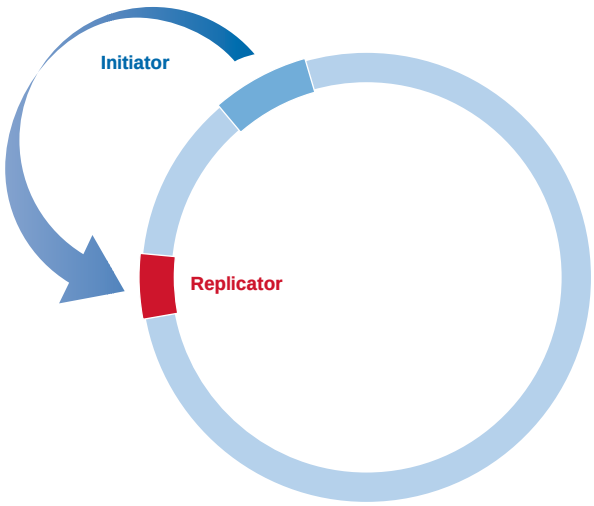

Fig 1 | The replicon model. A *trans*-acting protein encoded by the initiator gene was proposed to recognize a *cis*-acting sequence (the replicator) that controls the initiation of DNA replication in the replicon (Jacob *et al*, 1963).

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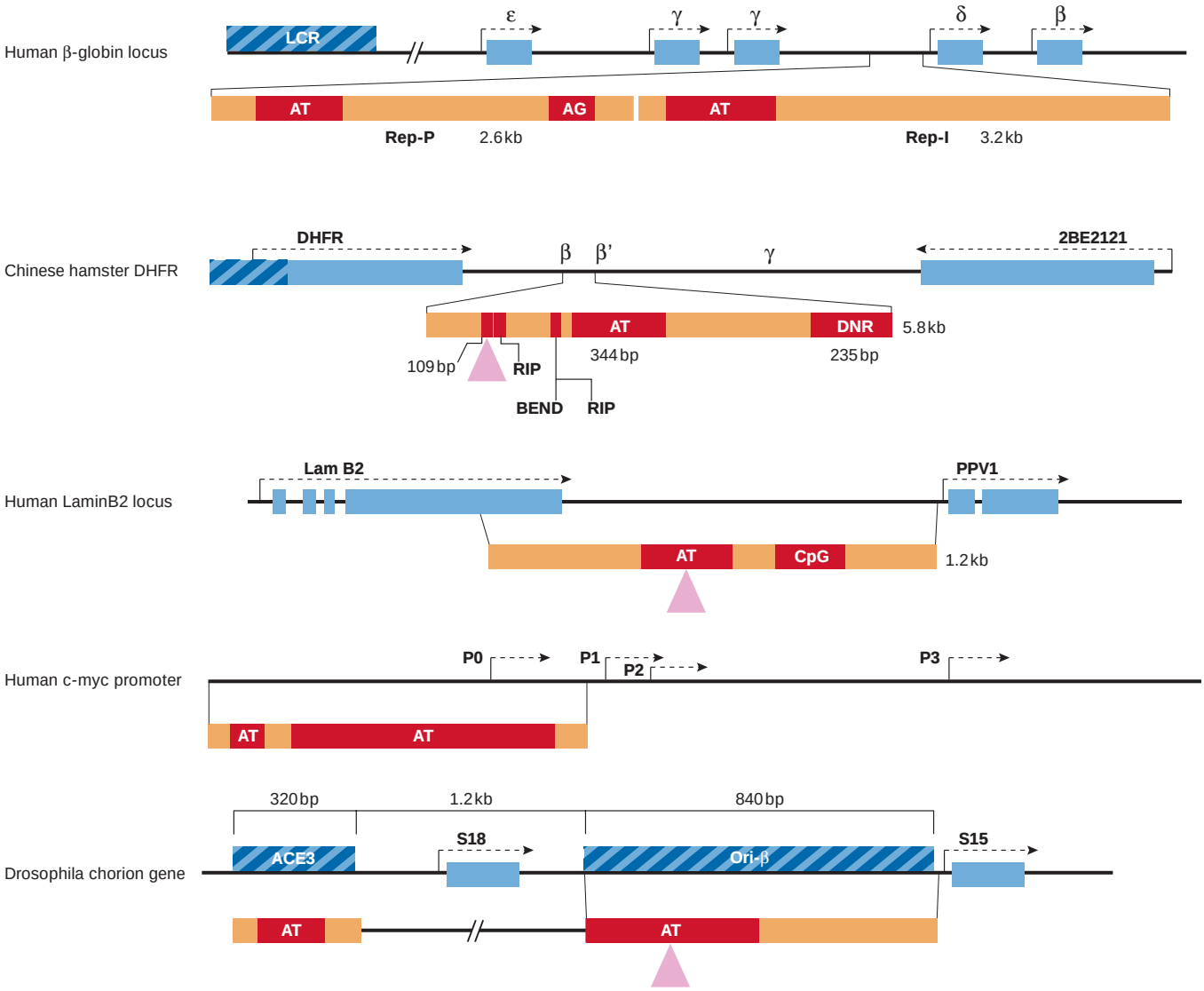

Fig 2 | Sequence features of metazoan replicators that are active at ectopic chromosomal sites. The genes surrounding the six replicators in five native loci are shown in blue (not to scale), and the elements required for initiation in the native locus are shown as hatched blue boxes. Mapped initiation regions in each replicator are indicated by purple triangles. In each ectopic replicator (yellow), the sequence elements in red have been shown to be required for full initiation activity. AG, asymmetric purine:pyrimidine regions; AT, AT-rich element; BEND, a sequence-directed bent DNA region; DHFR, dihydrofolate reductase; DNR, dinucleotide repeat; CpG, CpG island; LamB, lamin B; LCR, locus-control region; RIP60, replication-initiation region protein 60 kDa.

Will the real metazoan replicator please stand up?

Because the chromosomal context of a metazoan replicator might influence its ability to initiate replication at ectopic sites (see below), various strategies have been used to overcome position effects and reveal replicator activity. These measures include placing a series of replicator candidates at constant genomic sites (Aladjem *et al*, 1998; Malott & Leffak, 1999), placing them between chromatin insulators (Lu *et al*, 2001) and using pools of uncloned stably transfected cells to study replicator activity (Altman & Fanning, 2001; Paixao *et al*, 2004). The length of DNA shown to have ectopic replicator activity ranges from 1.2 kb (lamin B2) to 5.8 kb (dihydrofolate reductase (DHFR) ori-β).

Mutational analyses of these replicators identified DNA sequence elements that are necessary for replicator activity (Liu *et al*, 2003; Altman & Fanning, 2004; Paixao *et al*, 2004; Wang *et al*, 2004), but none of these crucial elements are able to specify initiation by themselves. For example, the initiation of DNA replication from the *Drosophila* chorion locus requires two distinct sequences: ori-β, which is a segment that overlaps the replication origin and includes two essential regions (140 and 226 base pairs (bp), respectively); and ACE3, a replication enhancer that contains an essential 142-bp sequence (Lu *et al*, 2001; Zhang & Tower, 2004; Fig 2). The Chinese hamster DHFR ori-β requires at least four elements for the initiation of DNA replica-

tion at ectopic loci (Altman & Fanning, 2001, 2004), and an additional 109-bp element cooperates with other sequences to initiate replication at ectopic sites and from the endogenous locus (G. Wahl & J.L. Kolman, personal communication). Similarly, the human c-MYC replicator requires several elements (Liu *et al*, 2003), and the lamin B2 replicator requires a 290-bp region and is enhanced by a separate element (Paixao *et al*, 2004). The human β-globin IR contains two non-overlapping independent replicators; initiation within each of these requires at least two distinct sequence elements (Wang *et al*, 2004). No consensus sequence has emerged from a sequence comparison of essential replicator elements.

These observations suggest that metazoan replicators are composed of several non-redundant sequence-specific modules that cooperate to direct local initiation. This apparently combinatorial organization of metazoan replicators is reminiscent of the control of metazoan transcription: a diverse collection of modules, which are dispersed up to several kilobases from the initiation site, collectively dictate where replication will initiate (Hochheimer & Tjian, 2003; Levine & Tjian, 2003).

Nuts and bolts: how do the replicator modules work?

The absence of a consensus sequence common even to a few metazoan replicators raises the question of how these replicators are recognized for the assembly of pre-replication complexes. In budding yeast, replicators exhibit a consensus binding site for the origin-recognition complex (ORC), which acts as a landing pad for pre-replication proteins. This site is an asymmetric AT-rich region (mostly A on one strand and mostly T on the other). No consensus was identified in fission yeast, but AT-rich stretches also serve as ORC-binding sites (Bell, 2002; Takahashi *et al*, 2003). Both symmetric (alternating A and T) and asymmetric AT-rich sequences are found in the DHFR ori-β, c-MYC and β-globin replicators (Fig 2). In the DHFR ori-β, deletion of a 344-bp AT-rich region, which contains both symmetric and asymmetric stretches, compromises ectopic initiation (Altman & Fanning, 2001). Other AT-rich sequences that are crucial for ectopic ori-β replicator activity include an intrinsically bent DNA that is dictated by five A_{3-4} stretches spaced at ten-nucleotide intervals, binding sites for the conserved polydactyl zinc-finger protein RIP60 formed by $(TTA)_{4-5}$ (Altman & Fanning, 2004) and a 109-bp region that cooperates with the other modules to dictate initiation (G. Wahl, personal communication). Similarly, AT-rich regions are necessary for ectopic initiation at the *Drosophila* chorion gene locus (Lu & Tower, 2001) and at the human c-MYC replicator (Liu *et al*, 2003). A long stretch of asymmetric AT-rich sequences is required for initiation at the human β-globin Rep-P replicator, but an evolutionarily conserved alternating (A and T) sequence is not (Wang *et al*, 2004).

Previously, AT-rich sequences were thought to serve primarily as DNA unwinding elements, but emerging evidence indicates a range of functions for these regions. For example, AT-rich sequences in the lamin B2 replicator bind ORC (Abdurashidova *et al*, 2003; Stefanovic *et al*, 2003) and can facilitate the loading of the Mcm4/6/7 helicase and the unwinding of duplex DNA *in vitro* (You *et al*, 2003). The two AT-rich elements in the chorion replicator (Bell, 2002) and the *Sciara* puff II/9A origin (Bielinsky *et al*, 2003) also bind ORC. The DHFR ori-β has two ORC-binding regions *in vivo*: one in the AT element and one at the start site for replication (A. Patten and E.F., unpublished observations). Interestingly, the ORC-binding region from the lamin B2 replicator can partially or fully substitute for the DHFR AT-rich element, but initiation occurs only at the ori-β start site (Altman & Fanning, 2004). Nevertheless, purified metazoan ORC shows no detectable binding specificity to lamin B2 and DHFR ori-β DNA *in vitro* (Vashee *et al*, 2003), which indicates that ORC recruitment to these AT-rich elements *in vivo* must require additional proteins and/or DNA elements. This idea is confirmed by the finding that ORC binds randomly to mammalian episomes that do not contain specific replicators, with some preference for AT-rich sequences (Schaarschmidt *et al*, 2004). The affinity of ORC for negatively supercoiled DNA is an order of magnitude greater than its affinity for linear or relaxed DNA, which points to an additional mechanism for ORC selection of DNA-binding sites (Remus *et al*, 2004).

Architectural roles for intrinsically bent AT-rich DNA can also be envisioned (Fig 2). The AT-hook DNA-binding motifs of fission yeast ORC4, which resemble those of the high mobility group protein HMG-I/Y, could have an architectural role (Bell, 2002). In the *Sciara* puff II/9A origin, the adjacent bent DNA region could act as a 'histone magnet' to attract histones to form nucleosomes over the bent DNA, leaving the origin free to bind ORC (S. Gerbi, personal communication). AT-rich bent DNA is commonly found in promoter regions and replicators of yeast, in SV40 and in prokaryotes, in which it is thought to facilitate duplex opening. Protein-mediated bending analogous to the HMG-I/Y-mediated DNA bending that facilitates V(D)J recombination, and the assembly and stabilization of transcription complexes at enhancers and promoters in eukaryotes, might also occur (Levine & Tjian, 2003). Replicators also contain binding sites for other proteins, such as RIP60 in DHFR ori-β (Altman & Fanning, 2004), DNA unwinding element-binding protein (DUE-B) and transcription factors in c-MYC (Liu *et al*, 2003; M. Leffak, personal communication), Hox proteins in lamin B2 (Stefanovic *et al*, 2003), and a MYB/p120-containing complex in the chorion replicator (Beall *et al*, 2002). Mutational analysis indicates that these binding sites contribute to replicator function, but their roles are not yet understood.

Asymmetric stretches of purines on one strand and pyrimidines on the other strand are found in several metazoan replicators (Fig 2). A TC/AG-rich dinucleotide-repeat region that can form triplex and Z-DNA *in vitro* is essential for initiation at the ectopic DHFR ori-β (Altman & Fanning, 2001). AG-rich sequences are present in the regions that are required for initiation activity of the two human β-globin replicators, and one of these asymmetric purine:pyrimidine stretches is essential for initiation (Wang *et al*, 2004). Similar, but not identical, AG-rich stretches (>75% AG) are present in the human lamin B2 replicator (Stefanovic *et al*, 2003). Whether the requirement for asymmetric purine:pyrimidine sequences is a universal feature of replicators, and its molecular significance, remain to be discovered.

The potential role of CpG islands in metazoan replicator function also remains poorly understood (Delgado *et al*, 1998; Rein *et al*, 1999; Paixao *et al*, 2004). Methylation of CpG islands is part of an epigenetic regulatory programme that leads to the silencing of specific genes in development and in the aetiology of diseases, such as cancer (Jaenisch & Bird, 2003). CpG methylation inhibits local ORC binding to chromatin and replication in

Xenopus egg extracts (Harvey & Newport, 2003). However, CpG methylation in the intergenic initiation zone downstream of the hamster *DHFR* locus enhances initiation at ori-β (Rein *et al*, 1999). CpG islands are not essential for initiation in the human β-globin (Wang *et al*, 2004) and the lamin B2 replicators, but inclusion of a CpG island enhances ectopic initiation from the lamin B2 replicator (Paixao *et al*, 2004). Further work will be required to understand how CpG elements and their methylation affect metazoan replicator activity.

Flexibility in origin choice

The wide range of elements that cooperate to determine replicator activity in genetic assays shows that many sequence combinations can serve as replicators. Why should the sequence requirements be satisfied in more than one way? Perhaps evolution has favoured a relaxed code for metazoan replicators to promote regulatory versatility. Analysis of single nascent DNA molecules from the Chinese hamster *GNAI3* locus provides strong evidence for such flexibility in metazoan origin usage (Anglana *et al*, 2003). The *GNAI3* locus contains a frequently used origin located 0.3-kb downstream of the gene, but it also contains some cryptic less-frequent initiation sites. Increasing the nucleotide pool led to a higher frequency of initiation from the primary origin, whereas reducing the nucleotide pool slowed replication forks and increased initiation at the secondary sites. The cell can therefore sense the rate of replication-fork progression and compensate by activating dormant origins in regions that otherwise would be replicated passively by forks originating at the primary origin. This versatility might also come into play when DNA damage stalls replication forks. Therefore, the Jesuit dictum that "many [origins] are called and few are chosen" (DePamphilis, 1993) provides some insurance against incomplete replication in metazoans, as well as in yeast, which could otherwise culminate in genetic instability (Bielinsky, 2003).

Interplay between replication and transcription

The initiation of DNA replication in the human β-globin locus requires a 40-kb region upstream of the globin gene cluster—known as the locus-control region (LCR)—that regulates globin gene expression (Aladjem *et al*, 1995, 2002, and references therein). Similarly, deletion of the transcriptional promoter at the native hamster *DHFR* locus prevents initiation in the downstream initiation zone, whereas replacement of the promoter by an inducible *Drosophila* promoter restores the original pattern of origin usage (Kalejta *et al*, 1998; Saha *et al*, 2004). These data strongly indicate that a distal transcriptional control element or transcription itself restricts origin usage in the downstream chromatin. Given that the distal sequences that are needed for initiation in native loci are dispensable for initiation in the ectopic DHFR ori-β or the β-globin replicator (Aladjem *et al*, 1998; Altman & Fanning, 2001), it seems likely that the requirement for distal elements can be satisfied by a range of sequences that can substitute for one another.

The pattern of replication start sites at a given locus can be programmed by development or tissue differentiation in coordination with changes in transcription. For example, in mitotic cells of *Sciara* larvae, replication in the *II/9A* locus initiates in a zone of 7–8 kb. When the *II/9A* locus undergoes developmentally programmed amplification and increased gene expression in salivary glands, the 3′ boundary of the initiation region contracts, which results in an initiation region of less than 2 kb. RNA polymerase associates with the new 3′ boundary and might help to define it (Lunyak *et al*, 2002). In another example, replication at the immunoglobulin heavy-chain locus in mouse non-B cells initiates at the extreme boundary of the locus. A unidirectional fork moves more than 500 kb to the 5′ boundary. In developing pro-B and pre-B cells, which are active in transcription and gene rearrangement, replication initiates earlier from latent origins that are not used in non-B cells (Zhou *et al*, 2002).

Replication through a locus might create a time window between replication-fork passage and nucleosomal packaging, which facilitates changes in higher order chromatin organization that might allow developmental and differentiation programmes to unfold. An example of developmentally programmed gene expression that requires DNA replication for its activation was recently elucidated in the *HoxB* locus of the mouse and *Xenopus* (Fisher & Mechali, 2003). Replication-fork progression through the *HoxB* locus seems to contribute to the collinearity of gene position and expression timing during development.

Global genomic analysis of metazoan replication timing and gene transcription indicates that early-replicating regions generally correlate with expressed genes, whereas late-replicating regions usually correlate with silent genes (Schuebler *et al*, 2002). Consistent with this correlation, the replication timing of monoallelically expressed immunoreceptor genes, olfactory genes, imprinted genes and X-linked genes in females is asynchronous: it is early for the active allele and late for the silent one (reviewed in Goren & Cedar, 2003). However, the genetic and epigenetic programmes that regulate replication timing remain poorly defined. Early-replicating chromatin is generally associated with acetylated histones, correlating with active gene expression, and experimental targeting of histone acetyltransferase activity to an origin advances its replication timing (Vogelauer *et al*, 2002). Consistent with this, an expressed transgene inserted into late-replicating mouse heterochromatin became hyperacetylated and advanced the replication timing of the flanking mouse sequences, whereas silencing of the transgene reverted to late replication (Lin *et al*, 2003). By contrast, histone hyperacetylation did not correlate with early replication at three of the four origins in the chicken β-globin locus, which shows that hyperacetylation is not necessary for early replication in all cells (Prioleau *et al*, 2003). DNA methylation, which is another epigenetic modification that often correlates with the gene-expression status of chromatin, also does not correlate with the timing of DNA replication (Gribnau *et al*, 2003). The picture emerging from these and other studies is that replication timing and origin choice are likely to determine, and be determined by, global chromatin remodelling coordinated with gene expression.

Gene expression is regulated in part by insulator elements that define chromatin domains that are looped out and held at their base by insulator-binding proteins anchored to nuclear lamins (Labrador & Corces, 2002). Similarly, 'replication factories' containing active replication enzymes at stationary sites on a nuclear scaffold in metazoan cells spool the template DNA through the factory, extruding the nascent strands into loops that are anchored at their base (reviewed in Frouin *et al*, 2003; Laskey & Madine, 2003). This higher order chromatin organization is also thought to facilitate the sequential assembly and disassembly of

hundreds of replication foci in S-phase mammalian nuclei (Sporbert *et al*, 2002). The similar spatial organization of gene expression and replication indicates that replicons might exist in chromatin loops that are defined by insulators. Consistent with this idea, *Drosophila* chorion gene amplification is position-independent when transcriptional insulators flank the locus, but amplification fails when the insulator is inserted between the two elements of the chorion replicator (Lu *et al*, 2001).

Cell-proliferation control and programmes of gene expression are intimately related to the spatial organization of replicons in the nucleus. Changes in the subnuclear spatial organization of replication foci occur in cells preparing to exit the cell cycle, and coincide with the recruitment of retinoblastoma tumour suppressor protein (RB) and histone deacetylases to the foci (Barbie *et al*, 2004). Consistent with this, binding of RB to the chorion locus in *Drosophila* is important in limiting developmentally regulated amplification at this locus (Bosco *et al*, 2001). Interestingly, RB recruitment to replication origins in human cells occurs in the same temporal order in which the origins are activated and is crucial in preventing endoreduplication after DNA damage in mammalian cells (Avni *et al*, 2003). These observations indicate that replication in the proper subnuclear location and temporal order might help to direct higher order chromatin structure, and to execute transcriptional programmes in proliferating and differentiating cells.

Conclusion

The replicator elements and initiator proteins proposed in the original replicon model are now recognizable in metazoan cells, but the range of sequence modules in metazoan replicators is much greater than anticipated. How these modules cooperate to recruit pre-replication complexes is a challenging question for future work. Histone modifications, DNA methylation, higher order chromatin organization and subnuclear position seem to regulate replication both temporally and spatially. However, the mechanisms that link replication patterns to programmes for development and differentiation remain to be elucidated.

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