MICROBIOLOGICAL REVIEWS, Sept. 1991, p. 512–542
0146-0749/91/030512-31$02.00/0

Vol. 55, No. 3

Relationship of Eukaryotic DNA Replication to Committed Gene Expression: General Theory for Gene Control

LUIS P. VILLARREAL

Department of Molecular Biology and Biochemistry, University of California at Irvine, Irvine, California 92717

CENTRAL THESIS: DNA REPLICATION AS BASIS FOR COMMITTED GENE EXPRESSION

A relationship between the division of metazoan cells and changes in differentiation state has been observed since the earliest times of experimentation in developmental biology (for a review, see reference 168). Various explanations for this relationship have been proposed (quantal mitosis by Holtzer et al. [166–168], replication-expression linkage by Brown and coworkers [41, 74, 154, 396]) but have not led to a generally accepted model for the participation of replication in differentiation (167, 168, 343). A similar relationship is seen with the DNA (but few RNA) viruses of higher organisms. Without exception, all known DNA viruses undergo a transition from early to late patterns of committed viral gene expression which involves or requires DNA replication. However, here too there is no general model to explain such a ubiquitous replication-expression relationship. A recent proposal (159) may apply to DNA viruses which code for their own accessory replication proteins, but it offers no general explanation of replication-linked commitment. A reexamination of the replication-expression issue is presented here, and more recent results in support of replication-linked expression are considered. This leads to a proposed general theory of gene commitment in which the initiation of DNA replication is proposed to be controlled by *trans*-acting factors. Replication control thus underlies the mechanism of eukaryotic gene commitment which uses stable chromatin structures and requires DNA replication for changes in committed state. This theory does not require positive or negative feedback loops to achieve stable expression patterns and contrasts sharply with those in which *trans*-acting factors can act dominantly through preexisting chromatin to reset gene commitment.

Current theories for the control of eukaryotic committed-gene expression are derived mostly from prokaryotic models. *trans*-acting transcription factors from both prokaryotes and eukaryotes appear to clearly direct promoter-specific and inducible transcription (100, 184, 235, 245, 256, 311). With the phage lambda model, changes in stable transcription patterns are thought to be achieved by dissociation of bound repressor molecules followed by association of different DNA-binding regulatory proteins (182, 300, 371). This action, coupled to positive and negative feedback loops, is proposed to provide the biophysical basis of the switching mechanism for stable gene commitment (333). Switching the state of gene commitment therefore requires free access to the regulatory DNA sequences by the specific DNA-binding regulatory proteins. Some theoretical problems with application of prokaryotic models to eukaryotic gene control have been noted (226, 253), leading some to propose that stable DNA-protein complexes may be needed to achieve the required specificity in the much larger eukaryotic chromosome (102, 374). A well-supported consensus exists, however, that *trans*-acting transcription factors must predominantly be involved in eukaryotic gene commitment.

THE PROBLEM: CHROMATIN STABILITY AND ACCESS TO REGULATORY DNA

Repressed Chromosomes and Chromosomal Domains

It has long been established that some eukaryotic chromosomes or regions of chromosomes are in a dominantly repressed state, inaccessible and unresponsive to *trans*-acting activators of transcription (for a review, see reference 395). This state is most apparent with the late-replicating inactive X chromosome, which remains repressed in the presence of an actively expressed X chromosome (for a review, see reference 155). A similar situation is also seen with heterochromatin domains of otherwise active chromosomes, such as the histone-repressed (189), late-replicating (291) *MAT* haplotype of *Saccharomyces cerevisiae* (for reviews, see references 4 and 189). Repressed chromatin is thought to be present in 30-nm filamentous structures in association with histone H1 (111, 185). Histone H1 is thought to be present at low levels in active chromatin (368). The propagation of a stable repressed DNA-protein structure through subsequent generations (67) has been observed and may underlie stable patterns of gene repression and epigenetic stability (163) such as seen with imprinting. Results with the *Drosophila* polycomb gene support this view in that stable chromatin structures appear to be involved in the lineage-specific repression of homeotic genes (285). Such states are insensitive to the presence of *trans*-acting factors and not dependent on feedback loops (4). Although direct structural modifications to DNA could also theoretically provide chromatin stability, the current consensus is that DNA modifications such as methylation (20, 72, 162) neither are universal nor appear to be decision points of differentiation but may closely reflect and perhaps further stabilize prior decisions due to other processes, such as the activity of *trans*-acting regulatory proteins (for a review, see reference 332). Thus, at the level of chromosomes and chromosomal domains, repressed gene expression seems dominant and these repressed domains seem inaccessible to the action of positive-acting transcriptional regulatory proteins.

Promoter Occlusion by Nucleosomes and Other Chromatin Proteins

Occlusion of promoters by bound nucleosomes also appears to be stable in vitro and refractory to factor competition (377; for reviews, see references 138 and 395). Stable DNA-bound nucleosomes are reported not to be displaced by high-affinity DNA-binding molecules such as heparin (295), nuclear factor 1 (NF1) (62), the glucocorticoid receptor (288), or the general transcription factor TFIID (400). There is a general consensus that nucleosomes will prevent

transcription in vitro when they occupy promoters (228, 400), but will not interfere with in vitro transcription by SP6 polymerase through a transcription unit (230). Nucleosomes may (230) or may not (229) be displaced by readthrough of polymerase (8) following initiation; although transient structural alterations without dissociation have been seen, these alterations may require DNA replication to reverse (208). In vitro DNA replication with T4 DNA polymerase also does not displace individual nucleosomes (28). Nucleosomes remain attached to DNA during synthesis but may partition onto one or the other daughter DNA molecule (28, 330). Taken together, these in vitro observations support the view that assembled chromatin which is transcriptionally repressed is a stable structure. In addition, genetic results with yeast mutants indicate that histones are nonspecific repressors of numerous TATA box promoted genes, which can be activated by prevailing *trans*-acting factors following inhibition of nucleosome synthesis and assembly (138, 395).

Stable Association of Transcription Factors with Chromatin

Eukaryotic transcription factors can clearly dissociate from naked regulatory DNA. These dissociation events can be readily observed in vitro and are the basis of numerous competition footprinting experiments (184, 235, 245, 320). Further assembly of these DNA-bound sequence-specific proteins with other generalized transcription factors or with nucleosome and other chromatin proteins, however, has generally resulted in structures (transcription complexes) which can no longer be inhibited by unbound factors and thus appear to have very low dissociation constants (22, 80, 130, 131, 145, 169, 188, 242, 301, 336, 375, 378, 397, 399, 400) (for an early review, see reference 41). Stable preinitiation transcription complexes were first reported with polymerase III transcription factor TFIIIA (130), but have more recently also been characterized for the generalized TFIID TATA box-binding factor, which is believed to be a limiting component for template commitment (378). Taken together, the above results indicate that both positive-acting transcription factors and negative-acting nonspecific nucleosome and chromatin proteins may be in stable structures (363). Such stable structures, however, pose a dilemma for promoter control according to prokaryote-based models, which require accessible DNA and use differential binding affinities to set gene commitment.

A POSSIBLE SOLUTION: DNA REPLICATION-BASED CHROMATIN ASSEMBLY

Several cellular processes which might offer solutions to the above dilemma of stable chromatin can be considered. One, a gene-specific process capable of removing repressive nucleosomes and/or replacing resident regulatory proteins with other gene-specific *trans*-acting factors, could exist. Although high-affinity binding proteins would seem good candidates, they have failed to demonstrate such a capacity, as indicated above. Alternatively, some gene-specific modification or degradation of bound factors might specifically clear chromatin or degrade bound factors. In general, there is little experimental support for the existence of such activities. The rapid loss of a single nucleosome from the glucocorticoid response element of the mouse mammary tumor virus long terminal repeat was observed following glucocorticoid induction (304). The transcriptional induction of this promoter, however, depends on the displacement of a bound nucleosome from an adjacent NF1 site, yet no nucleosome displacement has been observed in vitro (36, 288, 292). Others propose that stable template commitment may be involved in glucocorticoid induction in vivo (196). Additional steps may therefore be needed to achieve the nucleosome displacement observed in vivo.

The assembly of chromatin normally appears to occur during DNA replication (8, 120, 139, 364, 396, 398). Thus, DNA replication provides an opportunity to assemble newly committed chromatin (for reviews, see references 95, 363, and 395), avoiding the dilemma posed by stable chromatin. The long history of observations in developmental biology of an apparent relationship between cell division and differentiation would fit well into the view of replication-based assembly of stable chromatin (374).

Replicons as Units of Committed Gene Expression

It is proposed here that DNA replication (in replicon units) is the underlying basis for setting and changing committed patterns of gene expression. Functional chromatin stability is presumed, and newly replicated DNA is therefore the usual substrate for reconfiguring chromatin and changing states of differentiation. This implicates the initiation of DNA replication as the primary decision point during differentiation, because replication precedes and determines subsequent chromatin states. The two factors which must then be considered are how *trans*-acting DNA-binding proteins are involved and how stable chromatin is also involved in a replicon control mechanism. In addition, since all DNA replicates prior to mitosis, the cell type specificity of subgenomic DNA replication would appear to present a problem which must be addressed. A more general explanation is also offered to account for results which appear to argue against the involvement of DNA replication in differentiation.

trans-Acting Factors Associated with Origins of DNA Replication: Viral Models

How might *trans*-acting factors be involved in replicon-determined gene control? As reviewed by DePamphilis (87), most eukaryotic DNAs which are viral or cellular origins of replication or are autonomously replicating sequences (ARSs) in yeasts also contain binding sites for factors which are active transcriptional *trans*-acting proteins. In at least two situations, such amplifying sequences or ARSs appear to also correspond to chromosomal replicons (47, 48, 151, 381, 382). It therefore appears that eukaryotic origins are normally associated with *cis*-required DNA elements which may also be active for transcription, often as transcriptional enhancers. It is proposed that this association of *trans*-acting factor-binding sites with origins represents a general situation and that associated *trans*-acting factors control the initiation of origin-specific DNA replication. Such initiators appear similar to those proposed by Callan to account for the decreasing number of active origins used during development (51) (for a review, see reference 349). Evidence that *cis*-binding sites can control origin function was observed with the *Tetrahymena* ribosomal gene (205) and the *Drosophila* chorion gene (278, 279) and has been proposed for the mating type locus of *S. cerevisiae* (33, 96, 97, 337). The functional activity and sequence requirements for these *cis*-acting elements have been most extensively examined with the mouse polyomavirus and primate simian virus 40 (SV40). Polyomavirus DNA replication is completely dependent on cellular replication and chromatin proteins and is thought to be a good model for studying DNA replication

(88). It is proposed that these viral replication systems can be examined as models for the participation of *trans*-acting factors in the control of DNA replication. Some might question the use of viral models for replication control because it is often thought that "runaway" viral replicons bear little similarity to highly controlled cellular replicons. In fact, most if not all DNA viral replicons also appear subject to cellular (often cell cycle) control in various tissues as low levels of episomal viral DNAs are often maintained during persistent infections (this issue is considered further below).

Cell-Specific DNA Replication Controlled by *trans*-Acting Factors

Polyomavirus DNA replication in vivo absolutely requires an origin-adjacent enhancer, and polyomavirus appears to have two functionally juxtaposed enhancers (named A and B enhancers [92, 258, 298, 383]). These enhancers contain numerous binding sites for cellular *trans*-acting factors, generally considered transcription control factors (such as PEA1, a murine AP-1 [240]). Alterations of various cellular *trans*-acting factor-binding sites of both enhancers are associated with alterations in cell-specific polyomavirus DNA replication (56, 57, 89, 90, 92, 122, 214, 221, 234, 250, 258, 307, 383). Furthermore, the resulting cell-specific viral replicon is *cis*-restricted, and its replication will not complement replication of viral DNAs which have incorrect *cis*-regulatory DNA in mixed-infection experiments. This indicates that the expression of functional T antigen (T-Ag) does not alleviate *cis*-restricted viral DNA replication. Also, the transcription activity of the associated *cis* DNA is not essential. Cell-specific polyomavirus DNA replication can be directed by *cis*-acting DNA elements which may be subfunctional as transcriptional enhancers (309, 383), and this replication activity can be uncoupled from transcription activity (58, 383). Therefore, cell-type-specific early polyomavirus transcription is not sufficient to give cell-specific polyomavirus DNA replication. Because T-Ags are the only viral proteins needed for replication and do not alone direct cell-specific polyomavirus DNA replication, it is reasonable to propose that *cis*-restricted, cell-specific DNA replication itself may be a cellular process that is exploited by the small DNA viruses. Support for this view is seen with a seemingly related cellular situation, cell-specific amplification of the *Drosophila* chorion gene replicon during oogenesis. This replicon also requires an amplification control element in *cis* which appears to bind *trans*-acting factors (85, 279). Although it has yet to be established, a reasonable assumption is that most, if not all, metazoan origins may exhibit cell-specific replication activity as a result of binding sites for DNA-binding proteins, active for transcription.

With this view, it is now possible to offer an alternative mechanism for the action of *trans*-acting DNA-binding regulatory proteins in gene commitment. It is proposed here that these proteins can direct cell- and replicon-specific initiation of DNA replication which allows newly assembled chromatin to change differentiation states. The replication-differentiation activities of these proteins are, in turn, made more visible or apparent with some viral replicons.

The proposal that viral replicons are legitimate probes for cellular differentiation could be questioned. It should be noted that various other processes which were also first observed with viral systems (retrotransposition, oncogenes, frameshifting in translational control) were initially considered by many to be virus specific but later shown to be general. Viral systems have had good success as models. With polyomavirus there is considerable direct experimental evidence which support the legitimacy of polyomavirus DNA replication control as a probe for the regulation of differentiation. Myoblasts (10, 110, 233) and other undifferentiated cell types (86, 89, 90, 234) do not efficiently replicate polyomavirus until the cells differentiate. Selection of undifferentiated cells which cannot replicate polyomavirus also coselects for myoblasts which do not differentiate into myotubes. Thus, the two processes, cellular differentiation and polyomavirus DNA replication, appear to be genetically linked.

Stable Chromatin, *trans*-Acting Factors, and Replication Control: Implications from In Vitro Results

If it is accepted that *trans*-acting proteins can act to control the cell-specific initiation of DNA replication, how would a stable chromatin be involved in replicon initiation and subsequent gene commitment? Chromatin structure appears to be important for *trans*-acting factor activation of polyomavirus and SV40 DNA replication. Early results with T-Ag-dependent, origin-specific in vitro replication of naked polyomavirus DNA did not indicate a requirement for an enhancer (298). Yet in vivo replication absolutely requires an enhancer. This implies that chromatin components are needed for the enhancer-dependent DNA replication seen in vivo. Moreover, in vitro SV40 DNA replication can be made dependent on adjacent NF1-binding sites and the addition of purified replication proteins and NF1 (62). This NF1 dependence, however, requires prior coassembly of the DNA with NF1 into chromatin. NF1-dependent replication is not observed on naked DNA templates. Also, chromatin assembled without NF1 appears much less active for DNA replication even with subsequent NF1 addition. These observations are consistent with the proposal that *trans*-acting DNA-binding proteins affect DNA replication by creating stable structures. The specific mechanism by which bound *trans*-acting factors appear to make the origin available for replication proteins (241) is not known, although the participation of RNA polymerase is a reasonable possibility (156). RNA polymerase, however, does not appear to prime SV40 DNA synthesis (259). Also, cellular factors other than core histones appear to be important for chromatin effects on origin activity (141). This could be a general situation, but clarification awaits investigation with cellular origins. Even lambda DNA replication requires transcriptional factor involvement to initiate DNA synthesis when the origin is complexed with *Escherichia coli* histonelike proteins (252), implying that the issue of chromatin stability and origin activity may be very general.

Chromatin-resident *trans*-acting factors are thus proposed to control cell-specific replicon initiation and switch differentiation states by responding to signaling systems and specifically initiating replicon-specific DNA synthesis. The resulting chromatin is thereby reassembled with available *trans*-acting factors for subsequent potential gene expression. With this proposal, *trans*-acting factors are always bound to cognate DNA and need not dissociate or use positive or negative feedback loops. Stable chromatin is thus inherent in the replicon-based gene control theory and is consistent with observations, including asymmetric daughter differentiation, which could be due to asymmetric nucleosome and *trans*-acting factor segregation during replication (330). The available *trans*-acting factors at the time and place of chromatin assembly now become crucial for subsequent differentiation.

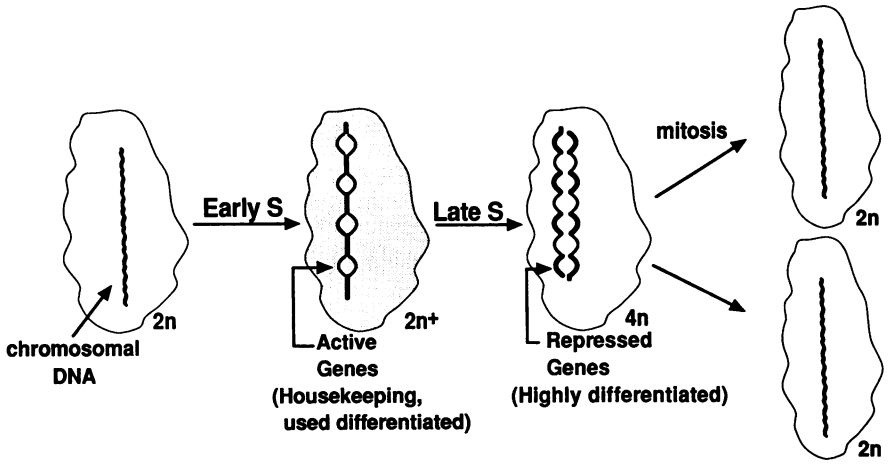


FIG. 1. General relationship of DNA replication to gene expression. The S phase of mitosis is shown to occur in two distinct periods, early S and late S. Most actively expressed genes, such as housekeeping genes, are replicated during the early S phase. Repressed genes replicate DNA during the late S phase as proposed by Brown (41).

Timing of DNA Replication and Prevalence of *trans*-Acting Factors

How, then, could replicons control chromatin regions with one setting of transcription potential distinct from other regions and settings? Since all DNA will replicate in mitotic cells, how can cell-specific replication differentiate subgenomic activated from repressed chromatin? The early observations of Taylor (367) and subsequent observations and proposals of others (41, 128, 130, 150, 164, 354), especially Brown and coworkers (41, 397), provide a solution. The time in the S phase of replication for a particular region of chromosomal DNA appears to be highly conserved (150) and to be related to its chromatin structure and transcriptional activity. Early-S-phase replication generally correlates with active euchromatic genes, and late replication correlates with repressed heterochromatic genes (41, 128, 130, 150, 165, 343, 354). Cytogenetic analysis indicates that all human chromosomes display distinct replication-timing chromatin patterns (54). It was proposed that DNA replication timing during this early and late biphasic S phase is directly involved in setting the transcription potential of replicated genes (41, 130). In these replication-expression models, transcriptionally active chromatin is established during early replication and transcriptionally repressed chromatin is assembled during the late S phase (128, 164, 165). This is shown schematically in Fig. 1. Replication timing and its relation to gene expression have been most widely studied with the 5S RNA genes of *Xenopus laevis* and globin family of genes (41, 53, 127, 140). With these 5S rDNA genes, it has been proposed that the somatic DNA which replicates early in the S phase is able to rapidly assemble with the prevailing and limited TFIIIA transcription factor and results in regions active for expression. This assembly is proposed to deplete TFIIIA so that during the late DNA replication of oocyte 5S DNA (41), chromatin assembles in the absence of TFIIIA with nucleosomes (336) or other chromatin proteins, such as histone H1 (327), into a repressed state. A similar situation is proposed for *Tetrahymena* rDNA (205). The loss of *trans*-acting factor function following the early S phase could be a general situation. The transcription factor OTF-1, which is involved in cell-cycle-regulated histone H2b expression, and NFIII factor can be isolated in active form only during the S phase and not during the G_2 phase (115, 273, 299), consistent with a cell-cycle-regulated inactivation of *trans*-acting factors. Late gene replication in *Physarum* species, however, appears not to preclude expression of all genes (290), so perhaps some *trans*-acting factors remain available for assembly with late-replicating DNA. If so, this would present a cumbersome situation in which positive and abundant negative factors compete for assembly onto DNA as proposed previously (9). Cell-cycle-coupled inactivation of *trans*-acting factor function, such as phosphorylation, might also be involved in differentiating the assembly of early- from late-replicating DNA.

trans-Acting Factors May Control Replicon Timing

The timing of DNA replication can apparently be affected and possibly controlled by *trans*-acting factor activity. Replication timing of mouse satellite (331) and integrated SV40 (236) DNA can be shifted from the late S phase to the early S phase depending on the cell-specific factors present (F9 cells) or the thermal stability of a temperature-sensitive large T-Ag, respectively. The chromatin-resident *trans*-acting factors may thus determine the timing of DNA replication.

By timing DNA replication, *trans*-acting factors residing on active replicons may set patterns of gene expression within the replicons, even though all DNA will ultimately replicate. This eliminates the apparent dilemma of achieving gene control with cell-specific DNA replication.

POSSIBLE MECHANISMS OF REPLICATION-PROGRAMMED DIFFERENTIATION

Control-Based Replication and Replication-Based Control: Implications for Genetic Programming

I have proposed three conditions of committed gene control which must be considered in determining how replicon-programmed gene expression might function: (i) chromatin is functionally stable; (ii) replicon timing is the basis of chromatin assembly and gene commitment; and (iii) resident *trans*-acting factors control subsequent replicon activity. Figure 2 incorporates the considerations from the viral

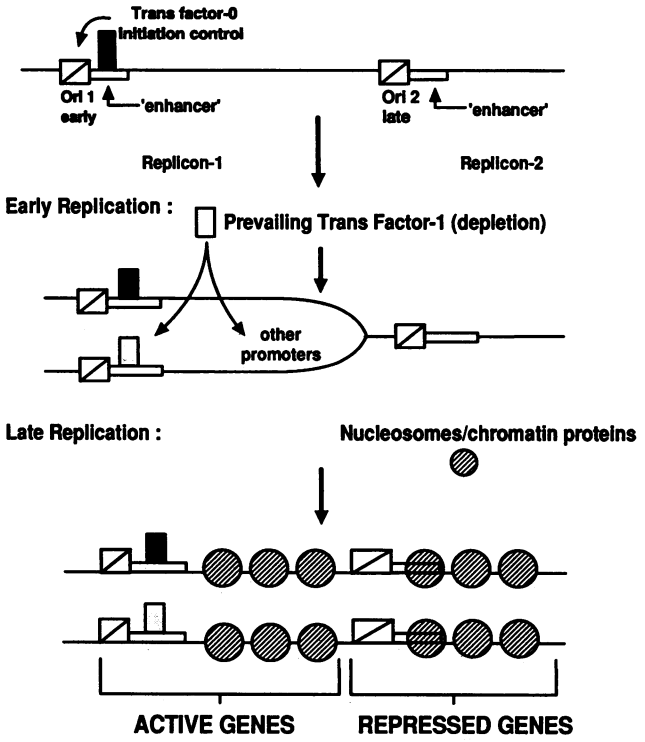


FIG. 2. Proposed participation of *trans*-acting factors in initiation of DNA replication and assembly of active and repressed chromatin. The early *ori-1* replicates during the early S phase under the control of bound *trans*-acting factor. Prevailing *trans*-acting factors can then assemble onto early origin and available promoters of daughter DNA to activate the replicon for possible expression. *trans*-acting factors may then be inactivated or depleted so that during the late S phase, late-replicating DNA is assembled into a repressed state with nucleosomes and other chromatin proteins. *trans*-acting factors need not dissociate from DNA.

models above and offers an alternative scheme for how *trans*-acting factors determine the states of committed gene expression. In Fig. 2, replicon 1 has a resident *trans*-acting factor 0, which allows early replication. Replicon 2 is repressed by nonspecific chromatin and/or nucleosomes and is late replicating. A second *trans*-acting factor 1 also recognizes *ori-1* but is coded for within replicon 1, which is expressed. Factor 1 is thus prevalent during early replicon 1 DNA synthesis and assembles with the daughter DNA strand. Any other binding sites (enhancers, promoters) for prevalent *trans*-acting factors within replicon 1 will also assemble into potentially active states. *trans*-acting factor 1 becomes depleted or inactivated. Late DNA replication then follows along with the expression of histones and other chromatin proteins. These replicons (and enhancers and/or promoters) assemble into promoter-occluded repressed states.

An implicit hierarchy exists among the three conditions noted which would affect programming models. The genes which code for *trans*-acting factors should themselves be available and active prior to DNA replication in order to be present for chromatin assembly. The expression of these new factors must therefore be from replicons which had been previously set for active gene expression during earlier rounds of DNA replication. Thus, there is a necessary linkage between replicons and *trans*-acting factor activity, and factors must be expressed from active replicons and be present prior to DNA synthesis, but bound factors also activate replicon initiation. Replicons beget *trans*-acting factors, and *trans*-acting factors beget replicons. Only following the decondensation and nucleosome replacement of protamine-associated sperm pronuclear DNA might chromatin assemble de novo without DNA replication (268). This otherwise general *trans*-acting factor-replicon relationship can lead to logical models which would allow lineage decisions to be programmed by regulatory DNA. In addition, although the mechanism proposed for programming is through replication origins and stable chromatin, it is nonetheless responsive to external signals by the sensing action of chromatin-bound, *trans*-acting factors. Such a genetic program would not have to rigidly count rounds of DNA replication to differentiate, but could await appropriate environmental signals (i.e., hormones) before recommitting the chromatin.

Experiments which examine possible programming linkage of *trans*-acting factors to replicon control are few. Interestingly, reports that the c-*myc* proto-oncogene may be a *trans*-acting factor for its own DNA replication could be relevant (172, 173, 246, 247, 319). Also, a proposed role for opposite DNA replication polarities in c-*myc* expression is of interest (212, 373) and perhaps generally pertinent to the relationship of replication polarity to transcription polarity (37). Others, however, have not repeated these observations with c-*myc* (142).

Replicon-Based Genetic Programming

It can now be considered how one replicon might be switched from a stable inactive state to a stable active state. The problem is to make late-replicating inactive DNA available for assembly with prevailing *trans*-acting factors. In Fig. 2, replicon 2 must become early replicating. In one possible scenario, replicons may be ordered and overlapping so that the replication of an active replicon can also invade and potentially activate an adjacent replicon. Early-S-phase DNA replication into the adjacent origin-containing DNA would also allow assembly of the second origin with prevailing *trans*-acting factors. The adjacent origin is now set for subsequent activation, assuming that the proper *trans*-acting factors (able to bind *ori-2*) were prevalent. This could allow a logical serial genetic program based on the overlap of sequential replicon domains. If, in addition to the above replicon arrangement, the genes for *trans*-acting factors that will activate subsequent replicons are coded within the currently active replicon, an arrangement such as that shown in Fig. 3 could result. In the example outlined (Fig. 3), three overlapping replicons are shown and each replicon codes for *trans*-acting factors which bind to and activate the next origin during early replication. Additional promoters within each replicon (100 to 300 kb) would also be expected to assemble for potential gene activity at this time. In such an arrangement, the position of a gene in a replicon is directly related to its developmental program of expression. Each round of replicon activation is dependent on expression of *trans*-acting factors from the previously active replicon. This may be a very orderly way to organize genes which are themselves regulators of development, as it requires a very specific order of activation of *trans*-acting proteins and replicons. Also, this arrangement is not dependent on competitive crosstalk from other prevalent *trans*-acting factors expressed by different active replicons.

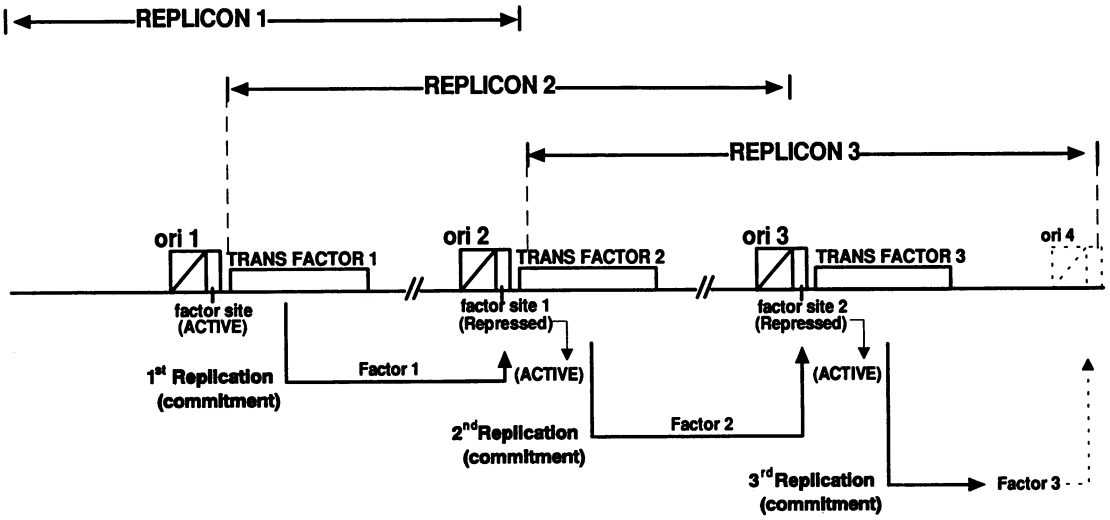


FIG. 3. Proposed control of genetic programming based on overlapping replicons. Shown are three overlapping replicons, each with its own origin of replication (*ori-1*, *ori-2*, and *ori-3*) with *cis*-acting binding sites (factor sites 1, 2, and 3) for binding *trans*-acting initiators. In the example shown, the gene for *trans*-acting factor 1 is within the early-replicating replicon 1. *ori-2* is "within" replicon 1 owing to overlap and allows assembly of *ori-2* with *trans*-acting factor 1. During the next round of division, *ori-2* is now early replicating. The process can be repeated to activate *ori-3* with *trans*-acting factor 2.

Predicted Organization of Developmental Genes

The above scheme gives rise to models which predict a direct relationship between genetic maps and developmental programming and serial genetic relationships between chromatin domains, *trans*-acting genes, and origins which affect replication timing. Features of this model are found in the genetic maps of the Antennapedia and Bithorax complex of *D. melanogaster* as well as the *Hox* and *HOM* loci of the mouse (3, 17, 186, 272, 287; for a review, see reference 325), and various other gene families such as globin (157). The homeotic genes are *trans*-acting regulators of development and are organized into serial arrays of gene family members whose positions in the genome are highly correlated with the time and position of their activity in development (2, 126, 281, 339). A replicon-based program provides an explanation of why the gene order is so strictly maintained during evolution as the gene position is directly related to its developmental programming. Other developmentally regulated gene families also appear to fit this organization and pattern of gene activation (immunoglobins, beta-globin [42, 134, 137, 154]). In addition, this model makes other specific predictions. The deletion of an intervening origin (such as *ori-3* [Fig. 3]) would fuse *ori-3* genes into the *ori-2* early replicon and should lead to the activation of *trans*-acting factor 3 (and other genes within replicon 3) during *ori-2* replication. This means that genes within the *ori-3* replicon would become activated one step ahead of their normal developmental program. This prediction is very much like what has been observed with the MCP and Fab-7 deletion mutations of the *Drosophila* bithorax gene locus (143, 186, 223, 224). Deletions of a small region of DNase-hypersensitive *cis*-regulatory DNA at the infra-abdominal boundaries (between *iab-4* and *iab-5* and also between *iab-6* and *iab-7*) result in transformations of posterior abdominal segments toward more anterior ones or premature gene activation, just as expected from overlapping replicon-based gene control. However, genetic results with viable *Drosophila* bithorax transpositions seem to suggest that genetic domain position is not needed for proper development (30, 272, 359). These results involve large segments of DNA and may be subjected to selection. Unselected transpositions of "complete" bithorax domains with flanking *cis*-active DNA have yet to be examined. Also, more pronounced transformations which could result from other transpositions (e.g., between *ubx* and *iab*) have not been observed (271a). Given the highly conserved nature of these genetic maps, these genetic results may not be a strong argument against a positional requirement for proper development. Another, possibly simpler, example of this overlapping replicon arrangement may be seen with the Epstein-Barr virus (EBV) genome. EBV may be a two-replicon (*ori-P* and *ori-Lyt*) version of programmed gene control in that it has two adjacent origins, each coding for its corresponding *trans*-acting factor and whose activation is correlated with origin activation and gene commitment (147, 361). Only *ori-P* (not *ori-Lyt*) is cell cycle controlled, which may indicate important alterations in states of replicon regulation (discussed further below).

Other Possibilities of Replicon-Programmed Differentiation

If a *trans*-acting factor is not inactivated during the early S phase and remains active during late-S-phase DNA replication, previously inactive replicons could assemble with them to become activated. Also, the polarity of binding sites for *trans*-acting factors may be involved in programming. Unlike transcriptional enhancers, *trans*-acting factor-binding sites for replication appear to be needed in a specific arrangement relative to the origin in order to activate DNA replication (55–58, 92, 241, 307). A distinct arrangement of binding sites could allow a program in which the sequential and/or accumulated expression of *trans*-acting factors is needed for subsequent rounds of replicon activation. In such a scheme, an increasing number of factors may have to be chromatin assembled to initiate subsequent rounds of DNA replication. Hematopoietic cell differentiation, in which there is an increase in numbers of growth factor required for each stage of differentiation, could be regulated in this way (314). Other possibilities, such as developmentally decreas-

ing origin availability or networks of *trans*-interacting replicons and *trans*-acting factors, could be considered, but there is little information which bears on these models. It is clear, however, that replicon-based gene control offers distinct strategies for programming committed gene expression compared with strictly transcription-based models and appears more consistent with the known genetic organization of developmentally regulated genes.

REPLICATION-DETERMINED DIFFERENTIATION REMAINS A MINORITY VIEW

The theory of quantal mitosis of Holtzer et al. was proposed early on to account for the involvement of DNA replication in myoblast differentiation. In it, there were crucial mitotic events which committed the resulting stable chromatin potential to subsequent differentiation (166–168). The idea of quantal mitosis is no longer favored as an explanation for myoblast terminal differentiation, yet clear correlations between specific times of DNA replication and differentiation continue to be made in the early development of *C. elagans* and other lower eukaryotes (6, 104). Some elements of the replication-determined differentiation model presented here were previously proposed by Brown (i.e., the replication-expression model [41]). Although this model appears to be applicable to some systems, especially multigene and proto-oncogene families (93, 94, 154, 176), a consensus that replication is needed for differentiation has not developed. Prevailing eukaryotic models have generally retained their biophysical (factor dissociation) basis from bacterially based transcription models (182; for reviews, see references 26, 75, 184, 235, 245, and 256) and have generally ignored the dilemma posed by chromatin stability, assuming, perhaps, that it is a separate control system. It is not intended here to challenge the ability of transcription models to explain promoter activation of assembled transcriptionally competent complexes. It is proposed here, however, that the commitment of transcription potential is made during the assembly of a stable chromatin prior to transcription and that former models addressed the activation or function of committed chromatin, not programming. These transcription-based models have endured because they provide a clear role for *trans*-acting factors which appears to be very consistent with many experimental results. Because replication-based models to date have not presented a more compelling case for the mechanism of action of these *trans*-acting factors in gene control or addressed the dilemma of stable chromatin and programmed gene commitment, they have failed to gain wide acceptance. I have presented the case for the participation of stable *trans*-acting and chromatin factors in replication-based gene commitment. Yet there remains apparently compelling evidence that DNA replication prior to terminal differentiation is in some cases absent (especially with myoblasts).

Results which Appear To Refute Replication-Based Differentiation

Results with myoblast terminal differentiation appear to refute the theory that prior DNA replication is required for gene commitment (63, 293, 376). During terminal differentiation of myoblasts to myotubes, inhibitors of polymerase alpha and delta (1-β-D-arabinofuranosylcytosine [ara-C] and aphidicolin) decrease [^{3}H]thymidine incorporation by 90 to 96% but do not prevent the differentiation of confluent myoblasts (23, 63, 294, 376, 402) and appear to actually increase differentiation rates (376) (unpublished observation). Myoblast differentiation does not appear to be a unique process. Expression of the *MyoD1* gene, which requires regions of similarity to the c-*myc* proto-oncogene for activity (105, 366), can actively differentiate various nonmyoblast cells (366) and is inhibitory to cell proliferation (344). Similar results are seen with *D. melanogaster* string mutants in that differentiation with resulting derepression of homeo-box gene expression occurs in the presence of aphidicolin (132).

Other results are more ambiguous in refuting the need for replication prior to terminal differentiation. Several cell lines, such as primary liver or HeLa cells, do not differentiate when expressing *MyoD1* unless they first replicate in the presence of 5-azacytidine (321). Also, addition of aphidicolin to subconfluent myoblasts in mitogen-poor media does prevent expression of muscle-specific genes (296), suggesting a replication linkage. However, if aphidicolin or ara-C is added to subconfluent dividing myoblasts, prior to terminal differentiation, the cells die instead of differentiating (unpublished observation). This indicates that the specific cell states or lineage may still require mitosis and therefore that possible stable chromatin structures may yet be important even for myoblast differentiation. Finally, ara-C and other inhibitors of DNA synthesis have differential effects on specific DNA replication origins, such as the *DHFR* replicon, which is relatively insensitive to inhibition and may still be reassembled into new chromatin in the presence of inhibitors (46, 209, 210). Altered chromatin assembly during inhibition of DNA synthesis has been reported (209, 211).

What, then, is to be concluded from these seemingly inharmonious results? Could several apparently dissimilar mechanisms of differentiation be operating in which replication is either involved (i.e., chromatin repression) or not involved (i.e., *trans*-acting factor activation)? I suggest that DNA replication may still underlie myoblast terminal differentiation even in situations in which the evidence against replication appears clear. The most compelling results against replication-based commitment were from Chui and Blau (63), who stated: "Although our methods were sufficiently sensitive to detect one-fifth of a round of replication, it could be argued that a minor amount of DNA synthesis occurred that was highly specific for the muscle genes. However, no precedents for localized DNA synthesis in the activation of genes have been described and the possibility seems unlikely."

Localized, cell-specific DNA synthesis is, however, clearly established in the case of the *Drosophila* chorion genes (279, 349, 350) as well as in most polytenized tissues (155, 187). Although these lower-eukaryotic examples with their endoreduplicated genome may not seem applicable to the situation being considered (vertebrate terminal differentiation), a generalized case can now be developed that all metazoan terminal differentiation may occur by a similar process involving prior localized or out-of-cell-cycle DNA synthesis.

TWO MODES OF DNA REPLICATION: MITOTIC AND TERMINAL CELLS

An Underlying Hypothesis May Explain Confusing Results

Although I have noted problems with the above experiments which argue against replication-based differentiation, certain observations are nonetheless clear and reproducible. In general, relatively specific inhibitors of DNA polymerases

alpha and delta (aphidicolin and ara-C [45, 197, 199]) have failed to prevent terminal differentiation in several important model systems. In some cases (e.g., HL-60 cells) these drugs actually induce terminal differentiation (136). Although I have implied that prior DNA replication may be gene specific, making such replication inapparent, this proposal is controversial as it counters the main point of several studies. What is the evidence for such inapparent replication, and why should it exist? Perhaps this issue could be more clearly developed if an underlying hypothesis is first stated and considered. It is here proposed that there are two basic states of metazoan differentiation, terminal and mitotic (or nonterminal), and that these two states have two correspondingly different modes of DNA replication control. I call these modes mitotic and terminal DNA replication and suggest that they differ in their linkage to the cell cycle. Mitotic replication occurs during the normal renewing cell division and is tightly cell cycle constrained. Terminal replication is not linked to the cell cycle but is needed for terminal differentiation. Terminal replication may at times replicate only replicons (genes) to be activated, representing a relatively small fraction of the genome. In addition, terminal replication appears to use some different replication proteins, which have distinct or lowered sensitivities to common inhibitors of DNA synthesis. It is proposed that this previously unrecognized mode of replication has often resulted in confusing experimental results, which appear to show that replication is not needed for differentiation. A further complication, presented below, is that most permanent or transformed cell lines have aberrant DNA replication control, and this has further added to the confusion of how replication is involved in differentiation.

Arguments and Evidence for the Existence of Mitotic and Terminal Replication Modes

What is the relationship between the control of the initiation of DNA replication and differentiation, specifically terminal differentiation? Normally, the initiation of DNA replication is tightly linked to the cell cycle. Renewing mitotic cells replicate each replicon once and only once per cell cycle in what may be one of the most rigorously controlled molecular processes (380). Such stringent control is necessary in systems which use thousands of replicons, as nonsynchronized replicons pose a potentially lethal problem of not maintaining a complete genome. Yet it is equally clear that there can be a type of DNA replication which does not require cell division. Some replicons can become uncoupled from the cell cycle (107, 254, 345, 347, 350) and continue replication without mitosis to yield polytene or polyploid nuclei. In this state cellular DNA clearly replicates without mitosis, nuclear breakdown, or cytokinesis by using a process called endoreduplication (for reviews, see references 39 and 40). Amplification of viral and some cellular DNAs (runaway replications) can also occur without a linkage to mitosis, as discussed below (306). In the great majority of situations, if not all, endoreduplication is associated with withdrawal from the cell cycle or terminal differentiation. Endoreduplication is specific to euchromatin domains and directly associated with highly expressed genes in terminal tissue (346). In *D. melanogaster*, euchromatin is generally overendoreduplicated whereas heterochromatin is either underendoreduplicated or not endoreduplicated (155, 346).

In lower eukaryotes, a relationship between endoreduplication and terminal mitosis also appears to be clear, as seen with the single-cell ciliates such as *Tetrahymena* species (for a review, see reference 21). These organisms have two nuclei, one of which (micronucleus) is diploid, is transcriptionally inactive, and divides mitotically in somatic cells. The other nucleus (macronucleus) has endoreduplicated as little as 5% of the genome, is transcriptionally very active, and either is unable to divide (in more primitive ciliates) or can divide amitotically for a finite number of divisions until the onset of senescence and cell death as a result of loss of genomic DNA sequences (11). All these types of amitotic DNA replication are similar in that replicons can reinitiate without mitosis. In this simple but perhaps fundamental sense, it seems clear that these are two recognizably distinct processes for the control of DNA replication: mitotic and amitotic. Also, the same replicon (i.e., rDNA [205]) may be subjected to both cell-cycle-linked and cell-cycle-unlinked modes of initiation control, and these two modes have distinguishable regulation by *cis*-active DNA.

Endoreduplication Precedes Terminal Differentiation in Dipterans

It is proposed that there is a basic and general relationship between terminal differentiation and these two modes of DNA replication. Cells which are mitotic are typically less differentiated (i.e., renewing stem, blast, and basal cells) than those which have endoreduplicated. The relatively small number of renewing diploid mitotic cells which are present in the Dipteran larva are destined to generate the imaginal disc and develop into adult tissues. Endoreduplicated cells are terminally differentiated and make up most of the adult and larval *Drosophila* tissue (for reviews, see references 150, 255, 313, and 328). The examination of polytenized *Drosophila* tissues may thus clarify or confirm the distinguishing features of these putative mitotic and especially terminal modes of DNA replication control.

The biochemical details of polytene DNA replication have not been fully elucidated. Early endoreduplication is cell type specific in that subregions which appear to replicate first correspond to regions which become active for gene expression (7, 349). This replication appears distinct from mitotic replication as injection of aphidicolin into early larva fails to inhibit endoreduplication (277a). This implies that endoreduplicated DNA synthesis either does not use DNA polymerase alpha or uses a polymerase insensitive to aphidicolin (318), such as DNA polymerase beta. It has been proposed that terminal differentiation or endoreduplication may involve polymerase beta-like activities (392). Polymerase beta is much less sensitive to both aphidicolin and ara-C than is polymerase alpha. Consistent with this is that mainly polymerase beta-like (with little polymerase alpha-like) DNA polymerase activity is found in either adult or larval *Drosophila* flies (318) which have predominantly endoreduplicating tissues. Yet, established (mitotic, nonpolyploid) dipteroid cell lines have substantial levels of DNA polymerase alpha and gamma (329). These results are consistent with the view that endoreduplication is a distinct process of DNA replication, involving polymerases (beta-like) other than polymerase alpha used for mitosis. Of some practical interest, endoreduplication may be as much as 20 to 50 times slower than mitotic DNA replication, as measured by using the *Drosophila* chorion gene amplification (351). Such slow synthesis, if generally true, could impair its detection.

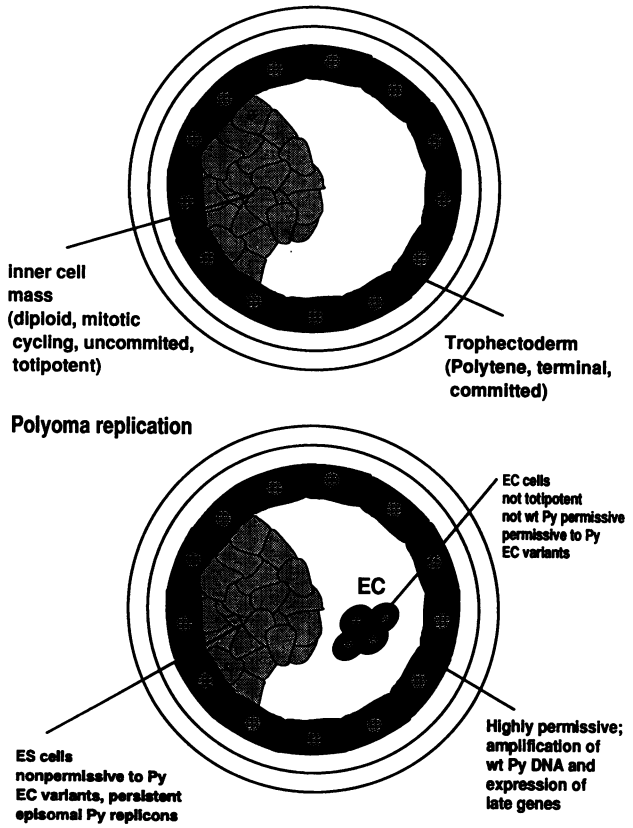


FIG. 4. First terminal differentiation of trophectoderm in a mouse blastocyst and the ability to replicate polyomavirus (Py). ES cells are totipotent cells derived from the inner cell mass. EC cells are related to ES cells but are omnipotent or nullipotent stable cell lines derived from teratocarcinomas. Wild-type polyomavirus replicates efficiently in trophectoderm but not in ES or EC cells, which become persistently infected. EC-selected variants of polyomavirus can replicate in some EC cells, but not inner mass cells. It is proposed that cellular mechanisms for replication control prevent polyomavirus DNA amplification in inner cell mass and allow polyomavirus DNA amplification in trophectoderm.

Mammalian Endoreduplication and Terminal Differentiation

What about mammalian systems? What evidence relates to the proposed existence of a distinct mode for DNA synthesis during differentiation or endoreduplication? Although polytene and polyploid tissues are widespread in lower eukaryotes (40) and such chromosomes appear to conserve common structural features from dipterans to protozoa (348), endoreduplication is not generally considered a common process in vertebrate development. Some polytene or polyploid mammalian cells are known, including megakaryocytes, cardiac muscle cells, and liver parenchyma (394), but the numbers of such tissues are limited. Yet polytenization and terminal differentiation are also linked in mammals and are crucial to their development. As noted by Kelly (191), Darwin (74a) first speculated that the earlier the stage at which a developmental mechanism functions during embryogenesis, the more strongly the mechanism tends to be conserved during evolution. We might therefore consider the first cells to terminally differentiate in the mammalian embryo in order to examine the relationship of endoreduplication to terminal differentiation. These first such cells in mice are the trophectoderm of the blastocyst (379). These first terminally differentiated cells are indeed a polytene cell type (15, 334) (Fig. 4). Thus, a conserved early evolutionary relationship of endoreduplication to terminal differentiation does appear to exist in early mammalian development.

Biochemical Studies of Mammalian Endoreduplication: Possible Role for Polymerase Beta

DNA synthesis in polytenized rat trophectoderm nuclei is reported to be insensitive to aphidicolin but sensitive to dideoxythymidine (338), a specific in vitro inhibitor of polymerase beta and, to a lesser extent, polymerase gamma, but not polymerase alpha (197, 257, 274). High concentrations of dideoxythymidine will also inhibit the postonset endoreduplication of trophectoderm in vivo (5), consistent with a role for DNA polymerase beta in endoreduplication. Polymerase beta was initially thought to be a repair enzyme because UV-induced unscheduled DNA synthesis is also inhibited by dideoxythymidine (99, 257) and is associated with increased DNA polymerase beta activity (118; for a review, see reference 392). Such repair synthesis is, however, sensitive to ara-C and aphidicolin (174, 175, 181, 257), indicating participation of DNA polymerase alpha but not beta. Also, following terminal differentiation of neuroblastoma cells when polymerase alpha levels are low and polymerase beta levels are high, repair of chemically modified DNA becomes inefficient (180), which also suggests the participation of polymerase alpha but not beta in repair. Terminal DNA replication, as I have proposed, is an amitotic, seemingly unscheduled form of DNA synthesis and would appear to be repair synthesis by many assays. Thus, terminal endoreduplication of mammalian cells may utilize aphidicolin-resistant beta-like polymerases.

Terminal differentiation in which endoreduplication is not thought to be involved may also involve DNA polymerase beta. Reports of lowered or absent polymerase alpha levels and constant or elevated polymerase beta levels in differentiated (but not undifferentiated) thyroid tissue (264, 265), nervous tissue (180), erythroleukemia cells (262), regenerating-differentiating rat liver (270), developing rat (270) or chick (243) brain, and differentiating chick lens (243) are consistent with a role of polymerase beta in terminal differentiation, distinct from mitotic replication, which is associated with polymerase alpha (243). A more direct assessment of possible DNA polymerase beta participation in terminal differentiation was recently reported by Zmudzka and Wilson (410). By using inducible expression of sense and antisense RNA for the polymerase beta gene, it was observed that antisense RNA-expressing cells increased their rate of doubling, but sense RNA-expressing cells completely stopped dividing after several divisions, yet remained viable, as if expression of elevated levels of polymerase beta leads to a terminally differentiated state. It could be very informative to examine specific inhibition of polymerase beta during differentiation in vivo. However, dideoxythymidine is phosphorylated inefficiently by cells, and other inhibitors (alpha and gamma interferons [365], human T-cell leukemia virus type I [HTLV-I] *tax* protein [178]) have not been examined. This issue needs closer examination, but current results are consistent with the proposed existence of a distinct mode of terminal DNA replication in vertebrates involving beta-like polymerases.

Why Terminal Replication May Be Incompatible with Cell Division

Endoreduplication can result in the replication of variable portions of the genome, and this replication can continue to reinitiate, replicate, and assemble into chromatin to form a polytene chromosome, all without mitosis. The presence of the resulting unsegregated daughter chromosome with unequal copies of DNA suggests why the cell may not be capable of continued mitosis and hence may be committed to a terminally differentiated state. A less evident feature of my proposal, however, is that all terminal differentiation, not just the more obvious endoreduplication, occurs by the distinct terminal mode of DNA replication, resulting in uneven levels of DNA. What is the basis for this more general proposal? The proposal stems from the premise that chromatin is functionally stable. Resetting a gene-specific region of stable chromatin to a very different and highly committed state is a similar problem for all terminal differentiating cells requiring DNA replication. Unlike for polytenization, however, it is proposed that most of the mammalian terminal replication need not amplify most of the genome, but need only replicate cell-specific (and gene-specific) replicons to assemble active chromatin. This subgenomic replication need involve only a small portion of the genome, reminiscent of more primitive ciliates, which endoreduplicate only 5% of their genome in the macronucleus (for a review, see reference 11). Thus, terminal (amitotic) DNA replication is proposed as a common process for all terminal differentiated cells, including polytenization, ciliated protozoan macronuclear endoreduplication, chromosomal and episomal gene amplification, and vertebrate terminal differentiation. This is summarized schematically in Fig. 5, and the various proposed common features of terminal replication are summarized below:

Occurs in highly differentiated cells
Not normally compatible with mitosis (i.e., terminal)
Initiation not constrained by mitosis
DNA synthesis not sensitive to aphidicolin or ara-C
Cell-specific DNA replication
May be active in most transformed cell lines
Corresponds to highly differentiated genes

Inapparent Terminal Replication in Vertebrates

We can now consider why the requirement for terminal replication has escaped previous detection in vertebrates. The following factors could have easily masked terminal replication in most experiments. Highly expressed genes or terminally activated replicons may constitute a very small fraction of the total DNA (well below 5%). Such small amounts of DNA replication would have to be examined directly to be detected, such as was done for the amplification of the Ha-*ras* gene in senescent fibroblasts (353). Second, terminal replication, like endoreduplication, may be insensitive to (and possibly induced by) the usual inhibitors of DNA replication and may involve a DNA polymerase other than polymerase alpha. Even if these drugs inhibit completion of terminal replication, they do not appear to prevent the initiation of DNA replication, since some origins of replication can be labeled in their presence (121) and can allow the reassembly of new histones onto *cis*-acting regulatory regions of DNA (121, 209, 211). Also, because terminal replication is proposed to be the first committed event of terminal differentiation, it could be missed if not specifically sought. With myoblasts, terminal replication may have already initiated when myoblasts become confluent. Furthermore, if rates of vertebrate terminal DNA synthesis are similar to *Drosophila* chorion gene amplification, this synthesis may be 25- to 50-fold slower than mitotic DNA synthesis, and most experiments could significantly underestimate the levels of terminal DNA synthesis (351).

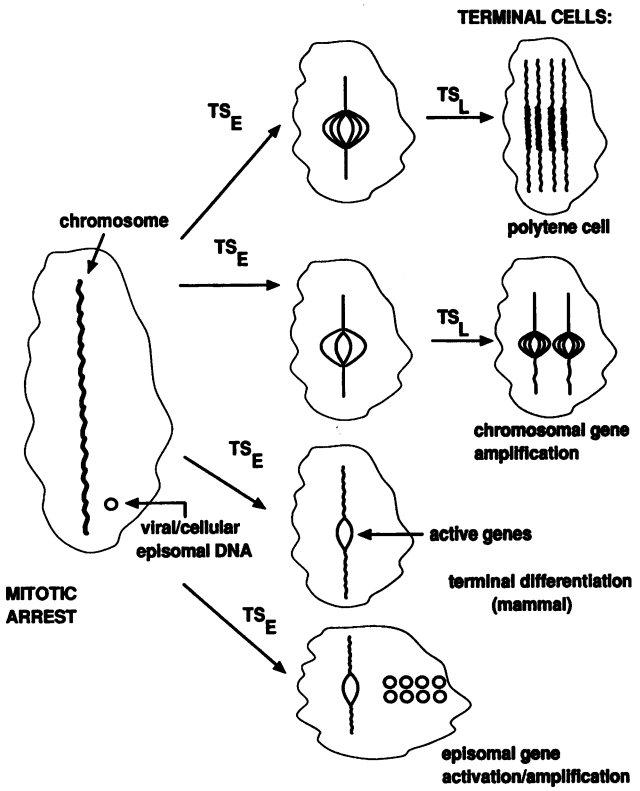


FIG. 5. General summary of the relationship of terminal differentiation to proposed terminal replication (endoreduplication). TS_E is the terminal early S phase. TS_L is the terminal late S phase seen in some polyploid or polytene tissues. The common feature is initiation of a specific origin of replication without complete mitosis.

The above arguments for vertebrate terminal replication are predominantly negative. Some recent positive evidence has been observed. The amplification of polyomavirus DNA in the presence of high levels of aphidicolin or ara-C in terminally differentiated (but not undifferentiated) myoblasts has been seen (88a). Also, aphidicolin-resistant cellular DNA labeled during myoblast differentiation (but not prior to differentiation) corresponds to discrete bands with restriction enzyme-cut cellular DNA (328a, 383a). Thus, aphidicolin-resistant, specific DNA synthesis is established in myotubes, but not myoblasts.

Chromatin in Terminally Differentiated Cells

A common problem of terminal differentiation in both lower and higher metazoan organisms is how to achieve the near-global activation of numerous highly expressed terminal genes while repressing many genes which were previously active in the progenitor basal or stem cells. A global process compatible with stable chromatin, such as terminal replication, offers a general solution to this problem but implies that a distinct chromatin state may result following terminal differentiation. What, then, is known about the

structure of chromatin in terminally differentiated versus undifferentiated nuclei? Although this has not been well studied, some differences have been noted. In *Tetrahymena* species, the endoreduplicated macronucleus does have more basic histone H1 than does the diploid micronucleus. Micronuclear H1 histones are made only in association with the micronuclear cell cycle (DNA replication), whereas macronuclear H1 histones (403) are made from an intron-containing mRNA not regulated by the cell cycle (404). In mammals, the core histones are made in tight association with the cell cycle, but expression of various H1 histone subtypes, such as $H1^0$ histone or the analogous avian H5 histone (216–220, 362), is not cell cycle controlled and their synthesis is directed by apparently stable poly(A)$^+$ mRNA (60, 61). These histones are expressed in various terminally differentiated cells but not their mitotic precursors (217). Thus, the regulation and type of histone production appear to differ in mitotic and terminal cells, consistent with the view that terminal differentiation may involve distinct chromatin states in which production of chromatin proteins is no longer tightly cell cycle linked. Also, the ability of the H5 histone to arrest cell proliferation and repress mitotic DNA replication (362) implies a terminal chromatin structure that may sometimes be incompatible with mitotic DNA replication. The rapid down-regulation of core histones H3 and H4 during terminal differentiation of myoblasts (206) would also be consistent with a major difference of terminal chromatin structure in at least these cells. That both SV40 and polyomavirus induce synthesis of histone 3.3 from a poly(A)$^+$ mRNA may be relevant to these ideas (171).

Other results may also be relevant. Terminal differentiation of erythroleukemia cells correlates with a c-*myc*-repressible $H1^0$ expression (61, 201). It has been reported that altered chromatin (depleted of H3 and H4) is assembled following hydroxyurea treatment of lymphoblastoid cells, which can induce terminal differentiation, and that these new altered histones are not mixed with old resident histones (211). That avian histone H5 DNA replicates in the opposite polarity in H5-expressing cells relative to nonexpressing cells suggests major changes in replication control during terminal differentiation (373). The generality, however, of this proposal that "terminal" histones or chromatin is involved in terminal differentiation has yet to be fully established. In addition, the role of such histones in endoreduplication is unknown. These results are, however, clearly consistent with the view that a major difference in chromatin structure occurs during terminal differentiation.

Viral Replicons in Normal Terminal and Mitotic Cells

The above discussion was focused on results and arguments supporting the existence of terminal replication. In general, terminal differentiation of mammalian tissue closely follows an asymmetric division of a basal or stem cell in which one daughter cell is committed to terminal differentiation with no apparent gene amplification (Fig. 6). I have proposed that such basal cells are in mitotic modes of DNA replication in which initiation of cellular replicon origins is restricted to once per cell cycle. However, many viral replicons which heavily depend on host replication and chromatin proteins appear to be runaway replicons able to amplify in dividing cells. It is proposed that in nondifferentiating mitotic cells, even runaway viral replicons will be chromatin constrained to replicate only once per cell cycle, just as cellular replicons are. In addition, viral regulatory proteins, such as T-Ag, should be unable to reset stable

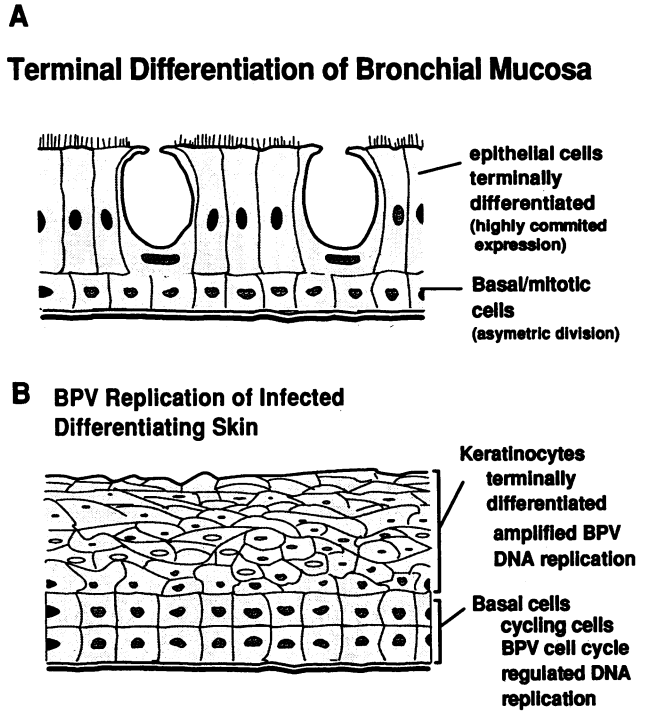


FIG. 6. Terminal differentiation of mammalian epithelial cells. (A) Architecture of normal bronchial mucosa in which the basal cell undergoes an asymmetric division to yield a terminal cell and a basal cell. (B) Replication states of bovine papillomavirus (BPV) in skin epithelial cells during terminal differentiation of keratinocytes. It is proposed that cellular replication modes restrict viral replication to be in either a cell-cycle-regulated state or a runaway state in terminal cells.

chromatin states to override this initiation control unless they can shift cells to the terminal mode of replication. Let us consider the evidence in support of this view.

Results with Polyomavirus

Polyomavirus DNA is not able to replicate as a runaway replicon in all cell types. It is proposed that the mitotic mode of replication control may be responsible for some of this cell type restriction. Early embryonic stem (ES) cells and other more committed but undifferentiated cell lines do not amplify polyomavirus or mouse retroviruses (Fig. 4) (1, 14, 213, 221, 369; for an early review, see reference 191). Instead, infection of these various undifferentiated cell types with wild-type polyomavirus will usually result in persistent maintenance of low levels of episomal viral DNAs (for early references, see reference 221). These cells include inner cells of a mouse blastocyst, various lines of undifferentiated pluripotent embryonal carcinoma (EC) cells (13, 73, 122, 146), normal embryo fibroblasts (221), undifferentiated myoblasts (110), undifferentiated erythroleukemia cells (86, 89), and neuroblastoma cells (89, 234). In all these cases, terminal differentiation of various cells leads to high-level wild-type polyomavirus DNA replication (replication with trophectoderm differentiation is shown in Fig. 4). In vivo, both mouse and hamster polyomavirus DNAs can amplify episomally or express late genes in various differentiated, but not basal, tumor cell types (76, 84, 120a, 232, 322, 323). Furthermore, in acutely infected animals, high-level replication in

both mouse and human polyomavirus (BK virus) are generally seen in terminally differentiated cell types, predominantly tubular epithelial cells (77, 269, 307, 308). These results are consistent with the proposal that undifferentiating mitotic cells superimpose cell-cycle-restricted DNA replication on polyomavirus genomes, whereas terminally differentiated cells allow runaway, amplified viral DNA replication.

One apparent problem with the above proposal (polyomavirus DNA amplification following terminal differentiation) is the amplification of EC cell-selected polyomavirus DNAs (e.g., F101 and F441 viral strains) in undifferentiated, apparently mitotic EC cells (122, 146, 250). Because EC cell lines are clearly populations of heterogeneous and distinct cells with different phenotypes and because even the EC-selected polyomavirus variants do not amplify in many of the individual cells in an EC culture, it is possible that some fraction of EC cells are undergoing lineage commitment, which allows the selected polyomavirus genomes to amplify. The relatively inefficient replication of EC-adapted virus in these undifferentiated EC cells compared with permissive or differentiated EC cells would be consistent with this view (56, 58). Also consistent is the fact that totipotent embryonal stem cells (inner mass cells [Fig. 4]) are not permissive to any of the EC-selected variants (249) but become permissive with differentiation (1). Thus, the results with the EC-selected polyomavirus strains are not inconsistent with the proposal that two distinct cellular replication processes may restrict or allow viral DNA amplification. In addition, it can be proposed that the ability to replicate these DNA viruses may actually illuminate or exaggerate the replication mode of the cell itself.

Other DNA Viruses: General Strategy for Persistent Infections

Papillomaviruses appear to conform to the predictions of mitotic and terminal replication modes. The episomal maintenance of papillomavirus genomes in basal epithelial cells is cell cycle restricted, whereas amplified papillomavirus DNA replication occurs with terminal differentiation of epithelial cells (411) (Fig. 6). This is consistent with the suggestion that viral replication may exaggerate the cellular mode of replication control. Perhaps related is the amplification of bovine papillomavirus and parvovirus DNA, unaccompanied by late-gene expression, in growth-arrested cells (49, 215, 405). A similar restriction of viral DNA replication in mitotic cells also appears to apply to the episomal maintenance (via *ori-P*) of EBV genomes in immortalized unstimulated B cells (148, 248) and in basal oral epithelial cells (91). In both these situations, amplification of viral DNA occurs (via *ori-Lyt*) only following stimulation or terminal differentiation of less differentiated (immortalized) mitotic cells. Cytomegalovirus may have similar dual replication control (12, 149). A clear inference is that DNA viruses may normally amplify in terminally differentiating cell types and be subjected to cell cycle control in undifferentiated progenitor cells. The episomal maintenance of viral replicons in undifferentiated basal cells may also provide a possible explanation for the ability of most DNA viruses to establish persistent infections for long periods in their immune hosts. If viral production is restricted to terminally differentiated cells, and if basal cells can maintain regulated episomes with little viral gene expression, the killing of these terminal cells by virus or the host immune response would not eliminate infected basal cells or prevent their subsequent differentiation with more virus production. Some apparent contradictions to these views concerning viral DNA replication in transformed cells are discussed below.

Amplified or Endoreduplicated Cellular DNA with Terminal Differentiation and Senescence

Some cellular genes undergo cell-specific amplification during terminal differentiation, indicating that this is not a strictly viral phenomenon. A well-studied example is the *Drosophila* chorion genes, which are amplified during oogenesis in ovarian follicle cells (85, 278, 279, 350). The chorion replicons are also dependent on *cis*-acting binding sites for *trans*-acting transcription factors and express high levels of chorion proteins following terminal differentiation. Thus, the existence of amplified cell-specific replicons in terminally differentiated cells is well established here. In addition, it appears that all transcription units within one of the two daughter DNAs of this replicon assume chromatin states compatible with active transcription (280), consistent with a direct participation of terminal replication in gene activation. Others have reported amplification or endoreduplication of DNA during normal senescence, including c-Ha-*ras-1* in normal diploid fibroblasts (353), increased DNA content in aging rat brain cells (360), and increasing polyploidy in aging rodent liver cells and human fibroblasts (39, 40). These last observations are most intriguing in light of suggestions by Goldstein that senescence appears to be very similar to terminal differentiation (129).

TRANSFORMATION AND REPLICON CONTROL

Abnormally Amplified Mammalian Replicons: Nuclear Oncogenes and Drug Resistance Genes

Some mammalian genes may also be amplified in a cell-specific manner, by what appears to be an abnormal process. Various tumor cells (especially lymphoid cells) have amplified assorted nuclear oncogenes and drug resistance genes (for reviews, see references 85, 324, and 356). A similar amplification of c-*myc* DNA has also been observed during the in vitro passage of HL-60 cells. Following various methods of growth arrest, these cells can amplify DNA and terminally differentiate to form monocytes (408, 409) in what appears to be a two-step process (406). In general the amplification of oncogene and drug resistance DNAs can be stimulated by arresting cell division or by transient inhibition of replication with various agents such as aphidicolin, ara-C, and hydroxyurea (170, 335). This effect, however, is not always observed (282). In other reports, a second round of early-S-phase DNA replication without mitosis is seen with growth arrest (144). In some instances, various genes will coamplify following inhibition of cell division, implicating some common process which may link their amplification (317). It is proposed here that such gene amplification is also due (at least initially) to replicon misfiring following growth arrest, which is characteristic of terminal replication (differentiation) as seen in HL-60 cells. Replicon misfiring which allows DHFR amplicon recombination and excision has been proposed previously (393), although subsequent accumulation of amplicons may occur by a recombinational mechanism (342). In most tumor cells, however, cell division can clearly continue even after amplification of these various DNAs. It therefore appears that this is an aberrant and perhaps basic feature of most tumors; they are mitotic, yet may be able to amplify (misfire) terminal replicons. Varsharvsky has proposed that replicon misfiring is important

for the emergence of malignant cell phenotypes (380), consistent with a mixed state of mitotic and terminal replication modes.

Transformed Cells and Aberrant DNA Replication Control: Concurrent Mitosis and DNA Amplification

The ability to amplify cellular DNA appears to be a major and dramatic distinction between transformed and normal diploid cells. Normal tissues from which these transformed cells are derived show no detectable frequency for gene amplification, yet amplification is far more frequent ($>10^8$-fold) in all transformed lines examined (370, 401). According to the theory of mitotic and terminal replication, such a difference in amplification potential signifies that transformed cells have lost the ability to prevent the reinitiation of DNA replication during mitosis and now allow the reinitiation leading to amplification of DNA, normally a characteristic of terminal differentiation. This further supports the view of concurrent mitotic and terminal replication modes with transformation.

Viral DNA Amplification in Transformed Cells

An important question then arises: why can various viral DNAs replicate to high levels (i.e., as runaway replicons) in cell lines in culture, since these cells are undoubtedly undergoing mitosis? If transformed cells do indeed have aberrant (mixed) replication control, their ability to amplify DNA and divide is not unexpected. We have examined the difference in cell type control of polyomavirus DNA replication in normal pancreas cells compared with transformed pancreatic cell lines (310) and observed that transformed pancreatic cell lines supported the full replication of wild-type polyomavirus, but we saw no replication in the pancreas in vivo, even though other polyomavirus enhancer variants can replicate in the pancreas (307). The transformed (mitotic) line thus displayed a relaxed control over cell-specific viral DNA amplification, even though these cells were expressing many pancreas-specific genes (276, 277).

Another issue is why aphidicolin, a specific inhibitor of polymerase alpha, can clearly inhibit SV40 and polyomavirus DNA replication in permissive cells in culture and in in vitro replication systems (81, 197, 259, 298) and why SV40 in vitro replication, unlike adenovirus replication, appears insensitive to dideoxythymidine triphosphate, a specific inhibitor of DNA polymerase beta (197). If terminal replication uses polymerases other than polymerase alpha, why are these amplifying systems sensitive to aphidicolin, in apparent contradiction to the involvement of terminal replication in DNA amplification? The in vitro systems have all been derived from transformed cells (mainly 293 and HeLa cells, both of which express early DNA viral proteins from adenovirus and human papillomavirus, respectively), so little can be said concerning the drug sensitivity of DNA replication in terminally differentiated cells. We have observed significant, albeit reduced, aphidicolin-resistant polyomavirus DNA replication in differentiating myoblasts (383b), yet it still appeared that early in infection (up to 12 h), polyomavirus was very sensitive to aphidicolin. It is possible that a virus-specific strategy is involved, in which viral genes such as T-Ag bind DNA polymerase alpha (125, 387) and use this bound polymerase for unconstrained DNA amplification in terminal cells.

Common Transforming Mechanisms Affecting Replication Modes

How might transformed cells allow both mitotic and terminal modes of DNA replication to occur? Which regulatory processes must be modified to achieve this state? Normal mitotic replication is proposed to rigorously restrict the initiation of DNA replication to only once per cell cycle per replicon, whereas terminal replication does not. It seems reasonable to propose that this cell cycle restriction must be eliminated to relax replication and growth control and that terminal replication must also be induced to allow the two modes to operate simultaneously. This further implies that some common regulatory processes must be perturbed for virtually all transformed cells.

The cellular p53 gene of vertebrates appears to be involved in cell progression from arrested to dividing states (303) and can immortalize cells (179) in mutant forms (161, 203). Most permanent cell lines and many diverse tumor types have a mutated form of their p53 gene (204, 222, 267). In addition, p53 (and the retinoblastoma gene, *Rb* [101]) is known to bind to SV40 and other DNA virus-transforming genes (large T-Ag). p53 competes for T-Ag binding to DNA polymerase alpha (125) and can be found associated (along with Rb) with cellular replication proteins at sites of herpesvirus DNA replication (391). Also, the murine form of p53 (but not mutant human p53) can interfere with T-Ag binding and origin unwinding in in vitro SV40 DNA replication (32, 387). The down-regulation of p53 during terminal differentiation (193) or Rb dephosphorylation in terminal (123, 202) or senescent (357) cells also suggests a role for p53 and Rb in the transition from mitotic and terminal replication modes. p53 and Rb are therefore good candidates to be the putative regulators of the mitotic mode and the transition to the terminal modes of DNA replication. Such regulators might initiate DNA replication while preventing replicon reinitiation within one cell cycle, as if to issue a "ticket" for origin usage. During terminal differentiation, this ticketing activity must then be properly inactivated to allow terminal replication. This could account for both the involvement in normal mitosis and the antioncogenic activity of wild-type p53. Wild-type p53 could suppress terminal replication proposed to be active in transformed cells and could possibly restore the normal cellular ability to terminally differentiate (114, 160, 161, 222). Such activities are consistent with alterations to common processes of replication control in all transformed cells. These views are also consistent with those of Harris (152, 153) and Stanbridge and coworkers (286, 355), who have proposed that tumor-suppressing genes may act to compel transformed cells to terminally differentiate, instead of growing indefinitely.

Terminally Differentiating Cell Lines with Normal Replication Control

Some immortal and primary cell lines, which are not fully transformed, do appear to maintain normal control (i.e., separation) of DNA replication modes. The ES and EC cell lines are examples of such a phenotype. These lines are derived from the inner cell mass of the blastocyst and are thus stemlike cells (Fig. 4) and are sometimes totipotent (ES cells [239]) for differentiation (66, 221, 312). Most EC cell lines and primary ES cell lines will divide indefinitely in culture as undifferentiated stemlike cells until they are induced to terminally differentiate following treatment with agents such as retinoic acid (19, 312). Differentiation pre-

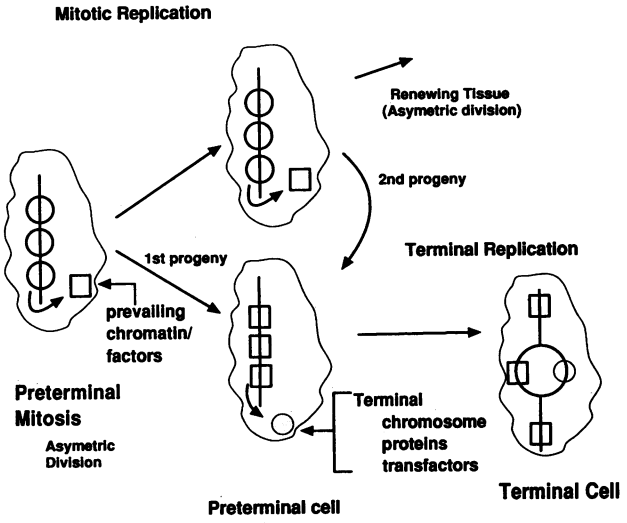


FIG. 7. Proposed transition from the mitotic replication mode to the terminal replication mode. A two-step asymmetric process is proposed to reassemble chromatin into the highly committed terminal state with prevailing *trans*-acting and chromatin factors. Resulting basal daughter cell can continue to generate a terminal progeny.

vents the further participation of these cells in embryo development (388). These lines differ from fully transformed cells in a number of important respects: they express highly differentiated genes only following terminal differentiation, they are generally nonpermissive to polyomavirus DNA replication (although persistent infections are often readily established [221]), they have a relatively stable diploid karyotype, and they express a wild-type form of p53 (114, 161). Resting B cells immortalized by EBV (148), primary mouse rodent brain cells (227), megakaryocyte precursors (394), and imaginal disc cells passed in adult flies (44, 271) may be similar in that they are all immortal, diploid, nontumor-forming cells, yet are able to differentiate. According to the replicon control model of committed gene expression, these cells appear to maintain the normal separation of mitotic and terminal modes of DNA replication and hence gene expression. Thus, immortalization per se may not necessarily affect replication control, aside from preventing an unprovoked transition to terminal states. Such cell lines may be more reliable models of normal DNA replication control than are the more transformed lines. We can now consider the common features proposed for the mitotic mode of DNA replication; these proposed common features of mitotic replication control are summarized below:

Replication occurs in less differentiated cells (stem or basal cells)
Replication occurs in mitotically competent cells
Initiation of DNA replication is once per cell cycle per replicon
DNA synthesis is sensitive to aphidicolin
DNA replicates in early and late S phase
Early-replicating DNA is potentially active for gene expression
Bound *trans*-acting factors determine replication time
Late-replicating DNA is usually repressed for gene expression
Bound nucleosomes and chromatin proteins repress gene expression
Mitosis and expression of terminal genes are normally incompatible

TRANSITION FROM MITOTIC TO TERMINAL REPLICATION

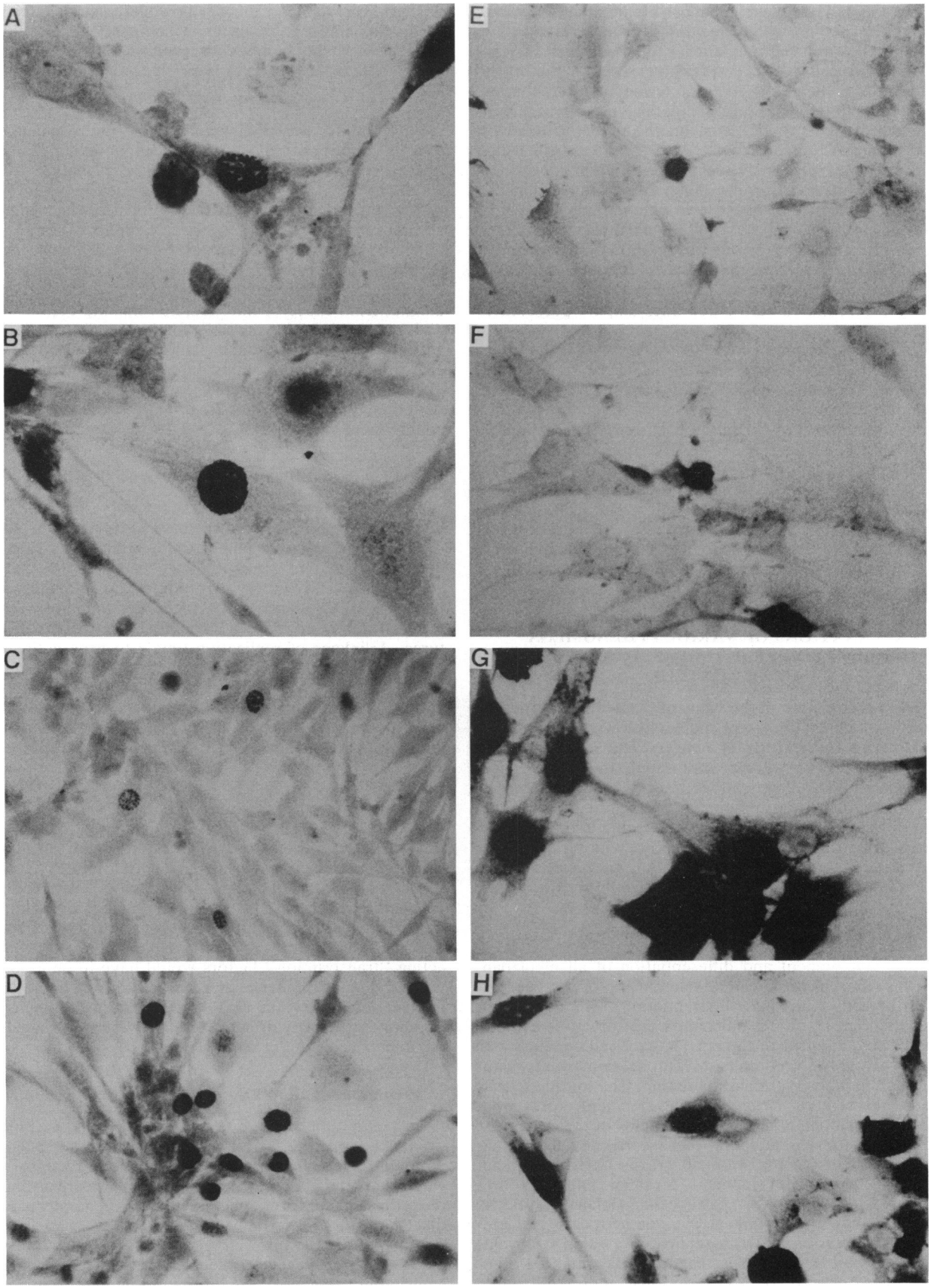
Two-Step Asymmetric Process

The earlier proposal for replicon-based genetic programming (Fig. 7) was applicable mainly to programming the lineages of mitotic cells (basal or stem cells) and hence proposed various ways of changing replication timing during the mitotic S phase. Terminal differentiation is often the cellular end product of such lineages. Since these cells can no longer divide yet must undergo major alterations in gene expression, this last transition in DNA replication and gene commitment may be rather different from previous mitoses. In renewing tissue, a basal cell will generally divide to yield two asymmetric daughters, one of which remains a basal cell, whereas the other, which is now growth arrested, will continue to differentiate. It is proposed that terminal replication must then initiate in this postmitotic cell to assemble the terminal chromatin structure. Thus, terminal differentiation should be a two-step process, involving a growth arrest precommitment and a postmitotic commitment. This two-step process is consistent with previous observations and proposals for terminal differentiation (61, 113, 201, 238, 305, 352, 406–408; for a review, see reference 112). A schematic of this process is shown in Fig. 7, in which terminal replication is included. The apparent ability of nucleosomes to remain attached to parental DNA during replication and segregate onto daughter DNA strands (28, 330, 389) could be the molecular basis of the asymmetric division, as only the daughter template may be terminally committed. This implies that the basal cell in this scheme does not actually regenerate but may be committed to produce only terminal offspring. This has interesting implications for the mechanism of senescence (129). Prior to cytokinesis of this committed basal cell, however, one would expect to have two nuclei in different replication modes (mitotic and terminal) in one cell. We have recent evidence for this prediction, as, during the terminal differentiation of myoblasts, keratinocytes, or primary embryo cells, polyomavirus DNA is seen to replicate in only one of the two nuclei in binucleate cells (Fig. 8) (13a).

Genetics and *trans*-Acting Factors of Terminal Replication

Little is known about the genetic and *trans*-acting factor requirements of putative terminal replication. With mouse polyomavirus in terminally differentiated myotubes and with embryonal carcinoma F9 cells, it appears that the binding sites for the ubiquitous AP-1-like PEA1 proteins and adjacent factor PEA3 (which bind the core sequences of the polyomavirus A enhancer) are primarily responsible for high-level viral DNA replication. These *trans*-acting factor levels are high in differentiated EC cells but not undifferentiated cells (110, 158, 198, 233). In addition, in vivo amplification of viral DNA in the kidneys, heart, and pancreas is

FIG. 8. In situ hybridization of differentiating C2C12 mouse myoblasts for the presence of polyomavirus DNA. A horseradish peroxidase method is used to detect polyomavirus DNA, which appears as dark areas (13a, 88a). (A to D) Accumulation of polyomavirus DNA in the presence of high concentrations of aphidicolin (up to 20 μg/ml) in differentiating myoblasts. (E to F) Some binucleate differentiating myoblasts in which only one of the nuclei is replicating polyomavirus DNA.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very efficient with two copies of the A enhancer and no B enhancer (308). Thus these enhancer-binding factors (AP-1/jun and c-*fos*-like) are good candidates for those involved in terminal replication, but they appear to be active in surprisingly different cell types. Expression of these factors in undifferentiated EC cells induces terminal differentiation (82, 83). Selection for the episomal persistence of viral DNA in undifferentiated mitotic cells, on the other hand, gives altered B, not A, enhancer (13). Also, persistence, but not amplification, of polyomavirus DNA in vivo appears to require B enhancer sequences (308). It is possible that the two polyomavirus enhancers are differentially active in mitotic (B enhancer) and terminal (A enhancer) replication. Although the generality of this observation will have to be extended to other systems, the control of terminal viral DNA replication could be rather nonspecific and may explain why so many differentiated mouse cell lines amplify polyomavirus DNA.

Another consideration is how to reassemble stable terminal chromatin from a repressed to an active state during replication. Terminal replicons may have to physically be within mitotic replicons to allow terminal origins to assemble for activity in the early S phase as discussed previously. Like the mitotic replicon-based genetic programming in Fig. 3, the overlap of mitotic and terminal replicons coupled to replicon-encoded *trans*-acting factors could be used to program the transition in replication modes. The EBV genome may be an example of such an arrangement in that *ori-P* (mitotic) and *ori-Lyt* (terminal) are juxtaposed and each codes for *trans*-acting proteins which affect replicon function.

EXPLANATIONS OF VARIOUS PHENOMENA ACCORDING TO REPLICON-BASED COMMITTED GENE CONTROL

The best assessment of the value of a new theory must be its ability to explain known phenomena as well as its ability to predict new experimental results. The replicon-based gene commitment theory appears to apply to a broad range of situations and offers rationalizations and predictions which are not otherwise apparent. Its basic premise, that the stability of committed gene expression is due to the stability of chromatin structures, is not in conflict with observations of chromatin or theoretical concerns about the mechanism of gene specificity and the size of the eukaryotic chromosome.

Replication-Differentiation Linkage

During development and differentiation of all metazoan organisms, there is an apparent relationship of a preceding division of cells with their differentiation. This linkage of mitosis to differentiation is most apparent during the earliest events of development of the egg as early cell commitment appears to involve predominantly replication events with little apparent transcriptional regulation. The mitotic fronts and domains of early *Drosophila* development correspond closely to developmental potential or fields of these regions (116, 117). Mutations affecting these early developmental events can affect the initiation of mitosis (103). Even the very latest event in development, terminal differentiation, typically follows mitosis of basal cells. Eukaryotic DNA viruses also show a close linkage of committed early and late viral gene expression to DNA replication, which is absent from virtually all RNA viruses. Theories of committed gene control based strictly on transcriptional action of *trans*-acting factors do not require a replication-gene commitment linkage. The replicon theory, however, necessitates DNA replication for changes in gene commitment and explains why this is such a highly conserved relationship.

Growth Potential and Differentiation

The great majority of metazoan cells which are committed to highly differentiated phenotypes cannot divide. Transcription-based models offer no general explanation for this situation. I have argued that a major resetting of chromatin occurs with a terminal replication process, which results in chromosomes with part (cell-specific replicons) or all (polytene) of the replicated daughter DNA remaining in the terminally replicated cell. I have further predicted that "terminal" chromosome-associated proteins may be quite distinct from those of mitotic cells, making continued cell division problematic, possibly fundamentally incompatible with further mitosis. Thus, a relationship between lost growth potential and a highly differentiated state is therefore expected. A relationship of senescence to terminal differentiation may also be consistent with replicon-based gene commitment (129).

Replication Timing and Gene Activity

The replication-repression model (41, 108, 128, 130), which has been previously proposed to account for the observation that active genes usually replicate early in the S phase and inactive genes replicate late in the S phase, is accepted here. My theory incorporates and expands on this proposal by explaining how *trans*-acting factors regulate replicon initiation and act through stable chromatin structures. A linkage of replication timing to gene activation is required by replicon-based gene control. There appears to be no reason to link replication timing to gene expression with strictly transcriptionally based models for gene control, since *trans*-acting factors are presumed to dissociate from the chromatin to allow transcription resetting.

Origins of Replication and *cis*-Linked Transcription Elements

The relationship between replication origins and binding sites for *trans*-acting factors is central to the theory of replicon-based gene control. Cell-specific DNA replication determines gene pattern commitment and is itself determined by these regulatory sequences and bound factors. There is no currently accepted explanation of why *trans*-acting "transcription" factors would have to be associated with origins (87). Hence the observation that most ARS domains of eukaryotes also contain binding sequences for *cis*-acting regulatory proteins or enhancers is expected from replicon-based gene control.

Programming Gene Expression: a Babel of Redundant Transcription Factors

I have proposed logical schemes for using replicons to program development and offer a plausible explanation of why developmentally regulated gene families are arranged in the order of their developmental use. It is generally accepted that regulatory DNA must contain much of the genetic information needed to instruct the genetic program for differentiation. However, recent results make it difficult to envision how some of this information could be organized,

or even specific, on the basis of transcriptional models for gene commitment. In the simplest situation, the binding sites for a single *trans*-acting factor appear to be a minimal unit of regulatory information for cell specificity. In a striking example with immunoglobulin-specific expression, it appears that E12 and E47 *trans*-acting factors bind to the kE2 DNA motif of the immunoglobulin light-chain enhancer and give cell-specific expression (251, 263). In addition, the same consensus sequence binds various MyoD-like *trans*-acting factors which are involved in the regulation of muscle-specific gene expression. However, B cells do not express muscle-specific proteins, nor do muscle cells express B-cell-specific proteins (251, 263). Furthermore, kE2 DNA binds and activates other MyoD-like proteins even though gene expression patterns of various muscle cell types are distinct (34, 35, 366). Thus, an enhancer motif appears to communicate different regulatory information to different cells. This suggests that the information content of important consensus sequences can be redundant or degenerate. How, then, does regulatory DNA instruct cell specificity of expression? If individual factors are specifying gene control but bind the same sequence, there appears to be a Babel-like situation with many genetic meanings for one consensus word. One proposal is that gene-specific inhibitory factors are also acting, apparently by binding as heterodimers, to either inactivate or alter binding specificities of prevailing *trans*-acting factors (183, 321, 366, 390). Also, the combination of binding sites might contain further information for cell specificity, although logical programming schemes are not obvious on the basis of these features. Also, kE2 DNA can act independently to activate lymphoid cell-specific transcription (289), implying that no combinatorial feature is needed. It is proposed that the redundant and confusing features of transcriptional regulation pose a dilemma for how regulatory DNA can program differentiation because no logical programming scheme is apparent.

The replicon theory of gene control offers plausible explanations of how a genetic program could be achieved by using redundant regulatory DNA elements. The use of redundant or degenerate binding sites for controlling tissue specificity is not problematic, because logical schemes (such as Fig. 3) use stable chromatin which is assembled only at specific replication times. Similar binding sites have the opportunity to bind only prevailing *trans*-acting factors following specific replicon activation. Thus, degenerate *trans*-acting factor-binding sites could still specifically set the chromatin for subsequent transcription patterns.

Difficulty of Isolating Mammalian Origins of Replication

Origins of replication are proposed to be under the control of cell-type-specific factors for initiation of DNA replication. This activity should be dependent on a number of factors, which include previously assembled chromatin structures, the specific prevalence and activity of *trans*-acting factors, the exact nature of the *cis*-regulatory DNA of the origin, and the mode of DNA replication (mitotic or terminal). This would make the task of isolating an active replication origin difficult, as origin activity would be dependent on all these features. In addition, if prior factor assembly is needed in some sequential, lineage-specific fashion, it may not be possible to properly assemble such structures from transfected DNA. Thus, replicon isolation may generally be very difficult, as has been noted previously (109, 151).

Contradictory Results and Two Replication Modes

Replicon-based control guarantees that some experimental systems will give apparently contradictory results. Because two replication modes are proposed which normally assemble distinct, possibly incompatible chromatin (terminal and mitotic), and because these two replication modes appear to also have distinct sensitivities to inhibitors of DNA synthesis, it should be possible to affect one but not the other mode of replication and resulting transcription. Controversy has persisted in the relationship of DNA replication to late-gene expression with the small DNA viruses. The replicon theory of gene control would view the early-to-late shift as a change in the replication mode from mitotic to terminal. Papillomaviruses, EBV, parvoviruses, and polyomavirus appear to fit this scenario, as differentiation of various mitotic cells is necessary for amplification of viral DNA and expression of late genes (for papillomavirus, see references 29 and 64). With the polyomaviruses, however, there are several reports that replication is directly required for late-gene activation (50, 68, 192), but is also incompatible with early-gene expression in adenovirus E1a-expressing 293 cells but not HeLa cells (207, 225). Others report that replication is unnecessary for late-gene expression in SV40 T-Ag-expressing COS cells (124, 190). A similar variable situation is seen with the dependence of beta-globin expression on DNA replication (16, 94, 108, 386). Although the chromatin structure has been proposed to be involved in replication-dependent late-gene activation (50, 364), no specific explanation of this involvement has emerged, nor have explanations been offered for the variable relationship of replication to late-gene activation. The replicon theory of gene control offers a plausible explanation for these apparently contradictory results. If late viral (or terminal) genes are normally activated following terminal replication and chromatin assembly, cells which have active terminal replication states (possibly most transformed cells) should appear not to require prior DNA replication, as newly transfected DNA will assemble into such a state. However, cells which are in the mitotic mode of DNA replication (possible situations include undifferentiated, primary, normal p53, and E1A-expressing cells) may express only early genes until viral DNA has replicated in a terminal mode. Thus, depending on cell states and on whether early or late transcription units are examined, contradictory results are not unexpected.

Terminal Chromatin, Bromodeoxyuridine, and Extinction

Terminal chromatin, as noted previously, may be rather distinct from mitotic chromatin. If so, this may offer an explanation for two long-standing phenomena that have not been adequately explained. "Extinction" of differentiated gene expression has often been seen when highly differentiated cells (e.g., hepatomas) are fused to less differentiated normal diploid fibroblasts (for a review, see reference 133). Mitotic diploid cells are expected to suppress unprovoked terminal replication and consequently suppress highly differentiated gene expression. Conversely, the ability of bromodeoxyuridine to suppress the expression of numerous genes characteristic of terminally differentiated cell types could be due to a general interference with terminal chromatin template assembly (31).

Gene Control in Normal and Transformed Cells

Various common features of altered transcription control in tumors have been noted (26, 194, 266, 283). Of specific relevance is the ability of most, if not all, tumor cells to express some genes characteristic of highly differentiated cells. Tumors are essentially phenotypically committed cells, similar in gene expression pattern to their normal mitotic progenitors, yet also usually expressing some highly committed genes. The replicon theory of gene control proposes that simultaneous mitotic and terminal replication could explain this mixed-expression state resulting from alterations of common control points of the initiation of DNA replication. This leads to inappropriate and general activation of lineage-available terminal genes. I have discussed how tumor suppressor genes may be involved in the control of initiation of DNA replication. A nuclear oncogene could inappropriately stimulate the initiation of terminal replication either as a *cis*-regulatory component (analog of T-Ag or *trans*-binding factors [AP-1/Jun, c-*fos*]) or as a component of the replication apparatus itself, assuming that tumor suppressors are also inactivated. The ability to suppress various tumor phenotypes by inducing terminal differentiation, with ara-C, retinoic acid, or other agents, would suggest that complete cellular commitment to terminal replication is still possible in some tumors (leukemias and embryonal carcinomas) (237, 312, 314–316, 388).

Activity of Endogenous and Exogenous Genes and Factors

DNA transfected into a cell should assemble with prevailing *trans*-acting factors and be expressed according to the activities of only those prevalent factors. If there are some regulatory activities which require *trans*-acting factors which are no longer prevalent, but were prevalent during previous mitosis, these lineage-dependent regulatory activities may not function correctly. A similar consideration would apply to the activity of *trans*-acting proteins introduced into specific cells. Their ability to affect the chromosome will be restricted to replicons which are already able to replicate in the early S phase, predicting that exogenous episomal genes could differ in regulation from endogenous chromosomal genes. Such a prediction might also explain the ability of transfected MyoD1 to initiate myogenic differentiation in fibroblasts but not in primary liver cells, since the latter cells may not have replicons that are active for myogenic differentiation (321).

REPLICON GENE CONTROL AND STRUCTURE OF CHROMATIN

Because the replicon-based theory of committed gene control makes specific and important predictions about the nature, structure, and function of eukaryotic chromatin, predictions applicable to chromatin are not examined separately.

Compatibility of Chromatin Domains, Gene Activity, and Replicons

Replicon-based gene commitment designates a replicon, not a transcription unit, as a unit of committed gene potential. As cellular replicons appear to be between 50 and 300 kb in size (150), numerous transcription units may reside within one typical replicon. Such transcription units should be assembled into potentially active chromatin or inactive chromatin as a unit of cell-specific DNA replication. Replicons are expected to have several components, including an origin of replication and *cis*-acting cell-specific regulatory sequences. Also, mitotic origins of replication are expected to be physically attached to structures, such as the nuclear matrix of membrane, which will allow segregation of daughter molecules (177). These requirements are consistent with known features of eukaryotic chromatin (for a review, see reference 157). Chromatin from mitotically competent cells is organized into structural and functional domains (18, 25, 69, 363) of about 50 to 300 kbp. These domains are in either condensed or extended structures, which correlate with inactive and active chromatin, respectively (164, 165). Junctions of heterochromatin and euchromatin are expected to be origins. Such junctions are typically DNase hypersensitive, associated with matrix, and often capable of ARS activity in *S. cerevisiae* (43). Position effect variegation, which represses gene expression in mosaic patterns of specific cells, is also associated with heterochromatin-euchromatin junctions and underreplication in *D. melanogaster* (155, 187). Each chromatin domain appears to correspond to a eukaryotic replicon (95, 128), consistent with proposals that the ends of domains are permanently attached replication origins fixed to nuclear structures (59, 98, 244, 284, 302, 340, 384). With the terminally amplified chorion genes, a domain also appears to correspond to a unit of transcription activation in one of the resulting daughter DNAs (280). All the transcription units within these domains are activated together, although here the origin appears to be in the middle of the domain. Inactivation of gene expression by using X-irradiation also suggests that the chromosome target sizes for expression are equivalent to chromatin domains, not smaller transcription units (231). Additionally, it has been reported that active genes (65), DNA polymerase alpha and beta (340), SV40 T-Ag (326), and even metabolic enzymes of DNA synthesis (297) are also associated with the matrix in a complex structure. Also of possible relevance is a gene-gating hypothesis of Blobel (24), which proposes that chromatin domains may be associated with or organized by nuclear pore complexes; this hypothesis has very interesting implications concerning the access of newly synthesized *trans*-acting factors to origins of replication. All of these proposed features of eukaryotic chromatin are schematically summarized in Fig. 9. A replicon basis appears to be consistent with the organization, structure, and function of chromatin. For the most part, these chromosome structures are not implicit in strictly transcription-based differentiation models.

Some Additional Predictions of Chromatin Structure-Function

Complete replicons with matrix attachment sites, origin, and *cis*-regulatory DNA should be *cis*-dominant for the cell-specific activation of transcription units within the replicon. Thus, such elements should be relatively position independent for expression and able to activate otherwise inappropriate promoters in a tissue-specific fashion. Consistent with this, the *Drosophila* chorion replicon genes appear to be expressed in a position-independent fashion (385). The dominant control region (also called the locus activation region [372]) of the beta-globin locus may also define such a replicon (134, 137). Dominant control region elements correspond to noncoding DNase-hypersensitive sequences which flank the beta-globin gene family locus. These flanking regions will confer erythroid cell-specific and chromosome

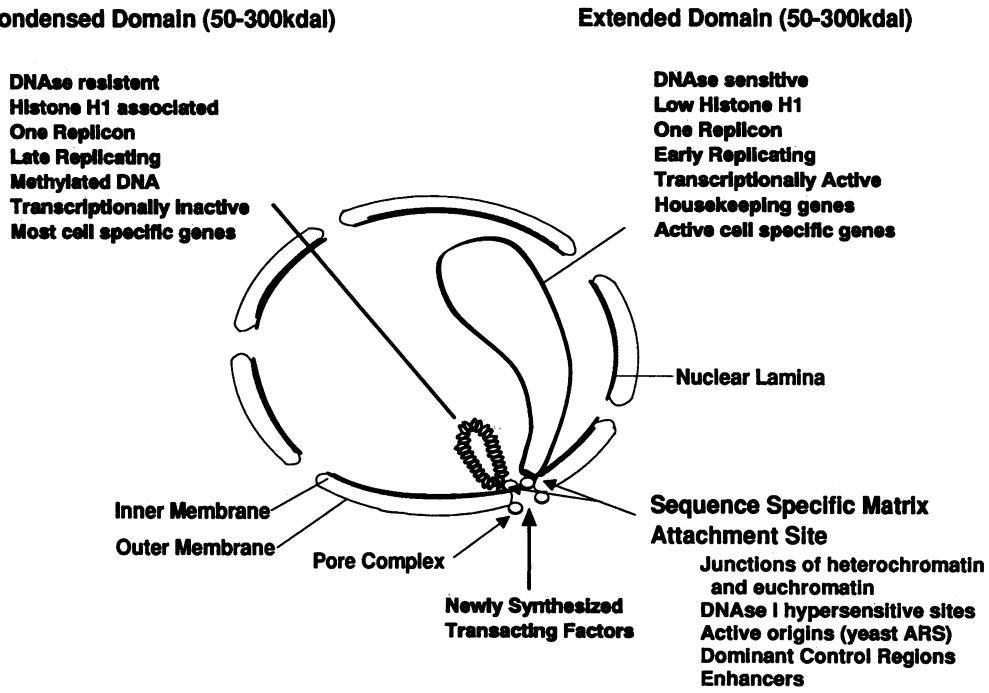

FIG. 9. Known and proposed features of eukaryotic chromatin. Shown is a schematic diagram of a condensed and extended domain of chromatin, along with the various features of structures which have been observed or proposed in the literature.

position-independent activation of even heterologous genes (157, 275, 372). The replicon theory of gene control predicts that the dominant control region elements should correspond to cell-specific origins of replication. Recent results of Forrester et al. show that locus activation region deletion renders the entire 100-kb beta-globin locus DNase resistant and late replicating in erythroid cells (119), in apparent confirmation of predictions. A report that chromatin attachment sites which flank the chromatin domain of the chicken lysozyme gene also confer chromosome position-independent gene activation to *cis*-linked transcription units is also consistent with replicon-based gene control (27, 358). Unpredictable tissue specificity of the introduced individual (nonreplicon) transcription units as a result of chromosome position effects (14, 283) are also expected.

Another predicted effect on chromosome structure would result from the process of terminal replication itself. Because cell-specific, out-of-cell-cycle replicon firing is proposed during terminal differentiation, there should be an accumulation of gene-specific replicon bubbles with associated DNA ends. These replicon ends would probably appear biochemically as nicks in specific regions of DNA and may also be fragile sites of the chromosome. Cell- and sequence-specific accumulation of DNA nicks following aphidicolin treatment of cells in terminal differentiation (but not in mitotic cells) has been reported with fibroblasts (261), resting lymphoid cells (106, 135, 200, 260), and differentiated myoblasts (79). Although the aphidicolin results might arguably be some type of drug artifact (frozen replication forks), these effects have been observed without drugs. These findings appear to support the prediction of terminal replication, but the generality of nick accumulation must be established.

EVOLUTION OF METAZOAN SYSTEMS: REPLICON VIEW

A question which has received relatively little consideration from a molecular perspective is the following: how did multicellular systems with terminally differentiated tissues evolve? Considering that all metazoans, even the simplest, with only two cell types (e.g., *Volvox* [195]), have a terminal (mortal or somatic) and nonterminal (immortal or germ) cell type, this appears to be a fundamental issue. The molecular issue is the evolution of highly committed gene expression, which is a characteristic of terminal cells. Beginning, presumably, from free-living individual cells, the transformation from the growth of colonies to tissue requires the recruitment of some of these cells to commit to specific patterns of gene expression rather than maximizing individual cell growth. A dead outer layer of cells may well protect inner cells of the colony from harmful effects of the environment (e.g., desiccation and UV irradiation), so some cell death may be beneficial to the survival of the colony. Could this seemingly altruistic relationship have been an early strategy which led to terminal differentiation? Other factors must also have been necessary. Most committed cells are highly active for gene expression, and cell death alone would not lead to such an active state. The emergence of relatively autonomous self-replicating DNA (replicons) in gene control, however, could lead to highly committed gene expression in nondividing cells. A replicon is expected to have an inherently selfish tendency to propagate, even at some cost to the host cell (78). If, however, replicons are also units of committed gene control, their selfish nature may be exploited by the organism and lead to committed patterns of gene expression in nondividing cells. By replicating at a lethal cost to the host cell, these replicons may establish a

terminally differentiated cell with a specific and high level of gene expression. This, then, offers an explanation for the evolution of terminal differentiation. The self-selecting feature of replicons means that they themselves, not only the entire organism, are subject to propagation and selection. Other genes (transcription units), beneficial to the whole organism, need only be within the replicon to give a cell a specific expression pattern. Such replication, however, should not be subjected to the same strict linkage to cell division; otherwise, amplification and propagation of DNA would be a problem. This general process could be repeated several times with different results, leading to the evolution of several distinct tissues whose development is controlled by very similar molecules and events. The genetic developmental pattern proposed in Fig. 6 might evolve from the overlap of such replicons.

I have proposed that a cell-cycle-restricted type of DNA replicon control is used for cell division and that a second type of replication is required for assembling chromatin, which allows highly committed gene expression. How could this second type of terminal replication evolve? In a sense, this appears to be the superimposition of one system of less regulated or runaway DNA replication on top of another, more regulated, multiorigin replication system. What entities now exist which might have been capable of superimposing a less restricted genomic replication onto the cell? Some type of rogue replicon which codes for its own polymerase and chromatin proteins would be a good candidate. The DNA viral genomes or cellular genomes with a single reinitiating origin, such as *E. coli*, appear to have these features. A virus infection, especially one with its own DNA polymerase enzymes and virus-specific chromatin proteins (such as adenovirus), could have been the evolutionary source of this terminal mode of DNA replication by participating in a symbiotic evolution with its host cell. This would be consistent with the symbiotic mechanisms proposed for the evolution of eukaryotic mitochondria and chloroplasts (341), but a more intimate molecular genetic relationship must have occurred to integrate terminal and mitotic replication. The dualistic nature of the eukaryotic chromosome, which has been proposed to account for the distinct structure and activity of housekeeping and cell-specific genes (128), could thus have resulted from such a molecular genetic symbiosis of two types of replicons. This dualistic chromosome seems to be a very early event in eukaryotic evolution. Even some unicellular eukaryotes, such as the protozoan ciliates, have dual nuclei, one of which can here be considered to be a mitotic nucleus and the other a terminal nucleus. The micronucleus contains the inactive diploid chromosomes used for sexual division, and the macronucleus has the active somatic terminal (senescent) chromosomes (403, 404). This may therefore be an extreme example of the structural segregation of mitotic and terminal replication modes and may also be relevant to the evolution of the sexual process itself.

SOME FINAL THOUGHTS

Biological theories which address global molecular strategies had good success during the early development of molecular biology. The proposed existence of mRNA and the adaptor hypothesis of tRNA are good examples of early successes, although the adaptor hypothesis was not published but only communicated to "tRNA tie club" members (71). Subsequent experience, however, with theories addressing global molecular strategies, such as the control of eukaryotic gene expression and development, were much less successful. These include theories for master and slave genes (52), gene batteries and repetitive DNA which activated otherwise repressed genes (38), and the involvement of unpaired single-stranded RNA in chromosome activation for gene expression (70). Instead, it appeared that most advances on these issues came from experimental, not theoretical, approaches. A common perception that appears to have resulted is that evolution is often inelegant and piecemeal in the way in which it solves biological problems. There appear to be many specific biological solutions to many specific problems, and thus theoretical solutions may not be globally applicable. The diversity of apparent biological solutions to problems appears to support this view. More recent experience, however, has shown a surprisingly large number of situations in which common molecular components are used in exceedingly diverse biological systems. Genes involved in signal transduction, cell cycling, differentiation, and oncogenic transformation are all good examples of this common-gene situation. These observations further imply the existence of common underlying molecular strategies which apply to these otherwise diverse biological situations. This view was the motivation for developing the replicon theory by committed gene control. It is believed that this global molecular model may be one of many underlying molecular strategies yet to be uncovered. Generalizations, such as the one presented here, will be necessary for integrating the vast amounts of information required to understand these biological strategies.

ACKNOWLEDGMENTS

I thank Roland Davis, Barbara Hamkalo, Suzanne Sandemeyer, Bert Semler, John Holland, Edward Wagner, Walter Eckhart, Michael O'Connor, Nicholas DePolo, and Meredith Peake for critical reading and comments on the manuscript. I also thank Juan P. Moreno for rendering the figures on an IBM personal computer.

This work was supported by Public Health Service grant GM 36605 from the National Institutes of Health; by the Organized Research Unit on the Molecular Biology of Animal Viruses at the University of California, Irvine; and by the University of California Cancer Research Institute.

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