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BrdU pulse/reverse staining protocols for investigating chromosome replication

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Abstract. By using a reverse Giemsa staining procedure (TT chromatin pale, TB chromatin dark) it is possible to detect replication in metaphase chromosomes with short (~10 min) 5-bromodeoxyuridine (BrdU) pulses. A pulse protocol allows us to consider the question "What is replicating at this point in time?" and we have investigated replication patterns during cycle transit in stimulated human female lymphocytes. A clear-cut demarcation between R-zone early and G-zone late was not found. Instead, whilst replication commences (with a very staggered start) in R-zones, activity soon appears to transgress band boundaries and gives rise to cells with unclassifiable patterns where chromosomes take on a mottled or reticulate appearance. Replication in R-zones dies out leaving a clear G-zone pattern persisting for the remainder of S which terminates with a very staggered finish. When pulse duration is increased (~1 h) the frequency of unclassifiable cells falls and occasional "mixed-pattern" cells appear which have, within the same cell, typical R- and G-zone regions. The existence of such cells indicates that if a mid-S replication pause exists (and the absence of any mid-S wave of pale stained cells suggests that it does not) it does not make exclusive separation between dark R- and G-band zones.

Introduction

The uptake of 5-bromodeoxyuridine (BrdU) during DNA synthesis coupled with subsequent differential Giemsa staining of bromouracil-containing chromatin is a widely used technique for investigating the programme of chromosome replication.

The usual protocol (e.g. Kim et al. 1975; Epplen et al. 1975; Cawood 1981; Camargo and Cervenka 1982; Savage and Prasad 1984) involves a "terminal" application, i.e. BrdU once added to the cell cultures remains until sampling. Chromosomes that were anywhere in S-phase when the BrdU was added arrive at the next metaphase with two types of chromatin: unsubstituted (TT) which was replicated *before* and substituted (TB) which was made *after* the BrdU arrived. An FPG-like staining method (Perry and Wolff 1974; Kim et al. 1975) renders TB pale and TT dark giving the chromosomes a banded appearance. The relative frequency of dark/light regions varies with transit of S-phase, cells near the start having few dark bands, and those near the end having predominantly dark chromosomes with a few pale bands. By using suitable sequential sampling regimes, the programme of replication at the level of the bands can be analyzed (Dutrillaux et al. 1976), and its regularity is such that it can be used to produce objective chronological sub-divisions in S which facilitate cell cycle analysis studies (Cawood 1981; Savage and Prasad 1984; Savage et al. 1984).

A terminal protocol results in a *cumulative* replication-band pattern, and whilst this has advantages for certain comparative studies (Savage and Papworth 1988) it has disadvantages when one wishes to study early and late-replicating bands within the same experiment. This is because the criteria for their identification differ, the former appearing as discrete dark bands in a pale chromosome, the latter as pale "gaps" in an increasingly dark chromosome. When such gaps are small, they are readily obscured and obliterated in contracted chromosomes, so that mapping late-replicating zones becomes increasingly unreliable as the end of S-phase is approached. Because short treatments with BrdU produce only small TB patches, the TB pale staining methods cannot be used in "pulse" regimes.

Late-replicating bands can be made to stain dark if a terminal thymidine protocol follows a period (several hours) of BrdU exposure. Regions replicating after the arrival of thymidine are now, of course, TT and stain dark, contrasting with the previously made TB chromatin (e.g. Grzeschik et al. 1975; Kondra and Ray 1978; Savage and Bhunya 1980). This method has been used

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for thymidine pulses (Schempp and Vogel 1978, 1979). However, terminal thymidine still does not allow one to look at early and late bands within the same culture, and it has a further disadvantage for experimental work since the necessary preceding BrdU incorporation can sensitize the chromosomes when certain clastogenic treatments are to be given.

An alternative approach would be to use a staining method that would render TB chromatin dark and TT chromatin pale. Several such "Reverse Harlequin" (RHQ) methods are available that can produce this reaction for TB/BB (Block 1982) and we have modified one of these (Alves and Jonasson 1978) to obtain a TT light/TB dark distinction with a terminal BrdU protocol. Such is the resolution and contrast available that we have also found the method quite able to detect pulses of BrdU as short as 5 min.

This allows us to answer the question "What is replicating at this point in time?" and so to investigate more thoroughly the central domain of S-phase about which relatively little is known, in particular the transition from early replicating R-zones to later replicating G-zones.

This paper presents some of our findings.

Materials and methods

Cell culture. Female blood lymphocytes, stimulated with phytohaemagglutinin (PHA), were grown in RPMI medium supplemented with serum, antibiotics and glutamine. BrdU (10 μg/ml final concentration) was added and remained in the culture until sampling for the terminal protocol. For pulse regimes, BrdU at the same concentration was added for 5, 10, 30 or 60 min, and then cultures were centrifuged and washed three times with non-BrdU medium and finally they were grown in conditioned media until sampling time. All manipulations were carried out in a 37° C hot room to avoid temperature shock. Samples were collected every 2 h from 2.5–18 h, following a 1 h Colcemid treatment. Air-dried metaphase samples were prepared and stained by the RHQ method (for details see Aghamohammadi and Savage 1989). Briefly, slides are stained in *freshly* prepared Giemsa solution (3 ml Giemsa to 40 ml of 0.3 M Na_2HPO_4 containing ~3% of 0.88 ammonia; the final pH should be 10.4). Following staining, slides are rinsed in tap water, blotted dry and mounted via xylene into Permount or DPX.

Scoring. We were unsure what patterns to expect from a pulse regime. Some previous studies had suggested an exclusive R-zone or G-zone pattern devoid of intermediates (Schempp and Vogel 1978, 1979; Schmidt 1980), and others that intermediate or mixed patterns are found (Dutrillaux 1975). Intermediate patterns were found and we therefore attempted to classify a random sample of *cells* at each sample time into the following categories:

Pale: No replication bands present – these cells would be pre- or post-S, and, if there is an absolutely quiescent pause during S, cells from this region.

R-zone pattern: The dominant replication bands present occupy *dark* R-zones. Cells may be further differentiated into *R-zone (pure) patterns* when there are no unclassifiable regions or *R-zone (impure)* when unclassifiable regions coexist within the same cell. These two types have been pooled for this report.

G-zone pattern: The dominant replication bands present occupy *dark* G-zones. *Pure* and *impure* patterns as above.

Unclassifiable: A cell, chromosome or region where the observed replication "bands" cannot be assigned with certainty to either an R-zone or G-zone pattern (Fig. 1).

Mixed pattern: Unambiguous R-zone and G-zone patterns within the same cell (Fig. 1). We have only found these with longer pulses and always accompanied by unclassifiable regions.

Partial harlequin (PHQ): At later sample times a few fast cells in second cycle are seen. These invariably have intermittent dark staining on one chromatid.

A note on the term "zone": in ordinary G-banding, the dark bands are characterised by relatively constant differences in size, staining intensity and position, and their number is more or less fixed in chromosomes of a given stage of contraction (ISCN 1981). The same is true for the pale bands, but the human eye is not fully aware of non-numerical differences until the staining procedure is reversed (R-banding).

In replication banding (and particularly when pulse protocols are used) *all* these characters can vary depending upon the position of the cell in S at the time BrdU is given (and the duration of the pulse). It is therefore not strictly valid to use terms like R-bands or R-band pattern etc. We have therefore substituted the term zone for a position on the chromosome which would ordinarily be occupied by a *dark* R- or G-band, and note that a replication band need not occupy the whole zone and, in certain cases, several small bands may occur within a zone.

Results

Terminal BrdU with RHQ staining

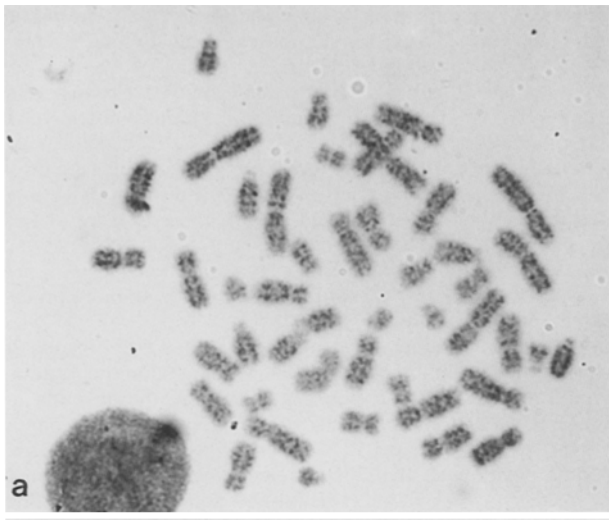
As a cross check that the RHQ staining method is detecting replication satisfactorily, a terminal protocol was run in parallel with the pulse regime. Since TB chromatin is stained dark, a G-zone pattern is observed throughout S. This is incomplete in cells towards the end of S, because G-zones terminate replication in an asynchronous, staggered manner. For most of S the pattern is complete, but as sampling time increases, some zones begin to merge giving, at first, solid-stained regions. These increase in size, leaving fewer and fewer pale (TT) gaps, which clearly occupy R-zone positions. Chromosomes become solid-stained only in pre-S cells.

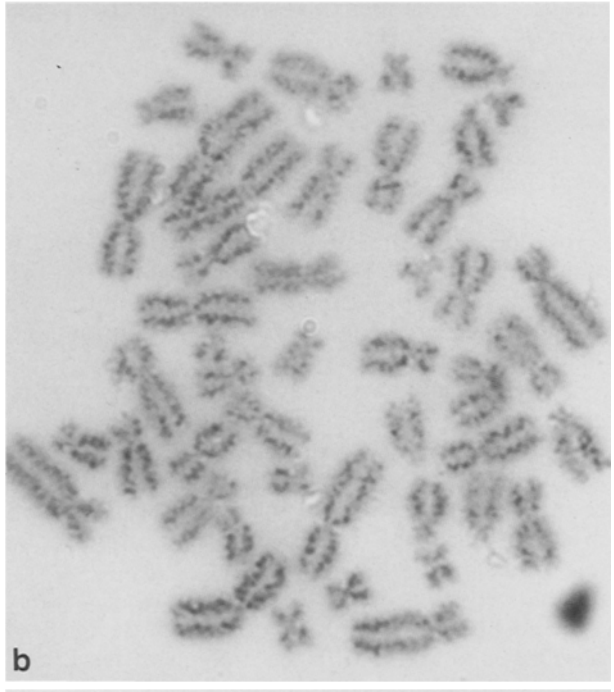
Qualitatively, this is the converse of KG staining (Savage and Bhunya 1980; Kim et al. 1975; Savage and Prasad 1984) and agrees with the findings of the BrdU/thymidine staining protocol (Grzeschik et al. 1975).

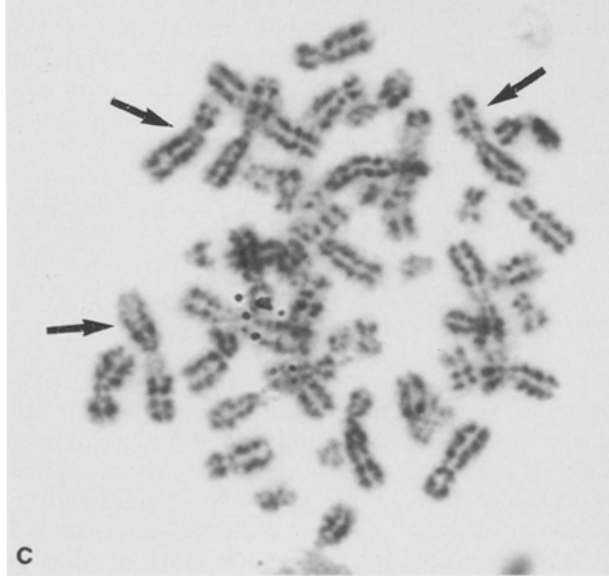
G-zone (pure) frequency bar charts for chromosomes 1–5 and X were constructed (Fig. 2) from random samples of single homologues at the 2.5 h fixation time (during the staggered termination of S) for both regimes. Apart from the major heterochromatic regions, where differences are to be expected, the bar charts were very comparable indicating acceptable efficiency of the RHQ method for pulse BrdU detection.

S transit mapped by short pulse

The resolution and staining contrast of the RHQ technique is such that we experienced no difficulty in detecting dark replication "bands" following a 5 min exposure to BrdU. However, analysis of metaphase chromosomes proved problematical, since many active zones and regions contained fragmented or multiple "mini-bands". This band fragmentation was much more obvious in







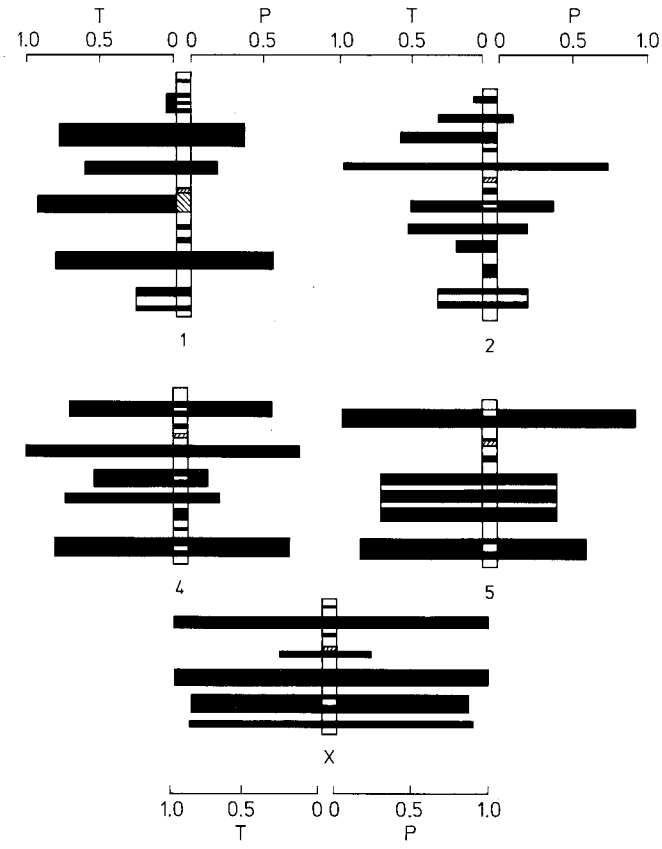


Fig. 2. Bar charts comparing the observed frequencies of replication zones observed at 2.5 h after the addition of terminal 5-bromodeoxyuridine (BrdU) (left) or after a 10 min pulse (right) for some lymphocyte chromosomes. The general similarities suggest comparable efficiencies of the two methods for detecting replication. The absence of bands after short pulses (e.g. 1*q* heterochromatin) indicates that the zone was not active or that replicational activity was below that resolvable with this pulse duration

early replicating R-zones than in later-replicating G-zones. Cell classification was uncertain or impossible, so we moved to longer pulse times where, although the difficulties did not disappear, they were sufficiently reduced to allow objective criteria to be used.

Figure 3 summarises the relative frequencies with sample time of various cell categories between 2.5–18 h when a 10 min pulse is used. The following features were observed. At earliest sample times (2.5 h, very late S) only two classes of cells are seen. Cells with totally pale chromosomes, devoid of bands, representing post-S (G_2) and cells with G-zone (pure) patterns, mostly incomplete, representing the staggered termination of S. By

Fig. 1. a, b Examples of "unclassifiable" cells which occur within S-phase when relatively short pulses are used. Some clear bands are seen but the majority of regions display a mottled or reticulate appearance so that the cell has neither a clear R-zone nor G-zone pattern. **c** A "mixed pattern" cell. Such cells are found only with longer pulses. *Arrows* indicate chromosomes 1, 5 and 3. 5*p* has the typical R-zone pattern with 5*q* tending to G-zone; 3*p* and 1*p* are typical G-zone patterns. Unclassifiable regions are also present

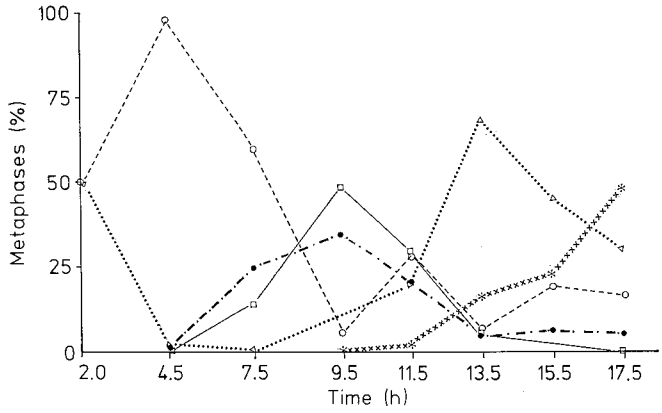


Fig. 3. Frequencies of the various cell categories during cycle transit in stimulated human female lymphocytes following a 10 min BrdU pulse and reverse (RHQ) staining. Note that the data are plotted to the mid-point of the 1 h Colcemid accumulation periods, i.e. 0.5 h less than the times stated in the text. Pale, non-S cells (△.....); G-zone pattern (○------); R-zone pattern (□———); unclassifiable cells (●-·-·-·-); partial harlequin, second cycle S cells* × × × × × × ×

5 h, nearly all cells have banded metaphases (G-zone [pure]) almost complete but many lacking the designated heterochromatin and various zones of the late-replicating X.

From 7.5 to 12 h, G-zone patterns begin to decline and some G-zone (impure) are seen. These latter have been combined with G-zone (pure) for Fig. 3. R-zone patterns, mostly impure, begin to appear and peak at 10 h. At these times, there is another group of metaphases present which we are unable to classify as either R-zone or G-zone. Most of these have a mottled or reticulate appearance transgressing band-zone boundaries (Fig. 1). These also rise to a peak at 10 h and then decline. In the first experiment we were not expecting such a class and when scoring, tended to force cells into an R-zone pattern. In later experiments (Table 1) with a 10 min pulse, we were more willing to recognise these as a distinct class and so their frequency, relative to R-zone patterns, is higher. It will be noted from Table 1, that as the pulse duration is increased, these unclassifiable cells are indeed mostly replaced by R-zone patterns.

S-phase has a *very* staggered start, and sample times from 10 h on have many R-zone (impure) and (pure) incomplete patterns present for a long period.

Cells in the second cycle since the pulse begin to appear after 10 h. These are characterised by discontinuous dark (TB) bands on one chromatid of an otherwise pale (TT) chromosome. We term these PHQ (Aghamohammadi and Savage 1989).

It is interesting to note that the replication progression deduced from terminal protocols is, in the main, confirmed with pulse. As G-zone patterns fall, R-zone patterns (together with unclassifiable cells) rise and these in turn fall as pale unbanded ($=G_1$ and G_2 of second division) and PHQ come in. It will also be noted that there are almost no pale cells in the mid-S region, and no sign of any "wave" of such cells during the period occupied by the first S.

Effect of pulse duration on cell classes in mid-S

The presence of a seemingly discrete cohort of unclassifiable cells in mid-S, led us to investigate the effect of increasing the pulse duration.

Table 1 shows the relative frequency of the different cell categories at two sample times spanning mid-S, after pulses of 10, 30 and 60 min. The frequency of pale cells remains low in all cases. As pulse duration is lengthened, unclassifiable cells fall significantly and G-zone slightly, whilst R-zone cells increase dramatically. Also, at longer pulse times, a new category appears, albeit at very low frequency, which is not found with short pulses. This

Table 1. The effect of lengthening pulse duration upon the relative frequency of the various cell categories

Sample time (h)	Duration of pulse (min)	R-zone	G-zone	Pale	Dark[a]	R and G mixed pattern	Unclassifiables	Total cells
7.5	60	82 (63)[b]	37 (28.5)	1 (0.8)	– 0	10 (7.7)	– –	130
	30	49 (40.8)	52 (43.3)	– 0	– 0	7 (5.8)	12 (10)	120
	10	24 (11.9)	88 (43.8)	5 (2.5)	2 (1)	– (0)	82 (40.8)	201
10	60	84 (64.1)	27 (20.6)	3 (2.3)	– (0)	– (0)	17 (13)	131
	30	33 (30)	20 (18.2)	6 (5.5)	8 (7.3)	5 (4.5)	38 (34.5)	110
	10	35 (13.5)	80 (30.8)	– (0)	– (0)	– (0)	145 (55.7)	260

[a] Unbanded, uniformly dark stained chromosomes, probably overstained pale
[b] Percentage in parentheses

is the mixed pattern having distinctive R-zone *and* G-zone regions within the same cell. Such cells are best seen by concentrating on two or more chromosome regions where the R- or G-zone patterns are characteristic and distinctive. Obviously, the observed frequency will be dependent upon the regions chosen.

Discussion

We have demonstrated that the RHQ staining method can be used to study replication and, in particular, that it is readily applicable to the detection and use of short BrdU pulses.

Duration of a pulse

One question needing to be raised is "How long is a pulse?", i.e. is there any significant incorporation of bromouracil after the BrdU has been washed out of the cultures? By significant, we mean that amount of incorporation needed before a resolvable dark dot can be seen.

Unfortunately, we cannot answer this question in absolute terms, but we do not think that significant incorporation goes on for very long after BrdU removal from the following considerations:
(a) There are clear distinctions in staining patterns and cell categories following exposures of 5 and 10 min. Both times are ample to flood the precursor pool given a concentration of 10 μg/ml.
(b) With shorter pulses we obtain discrete bands at all stages of S and these patterns change as cells transit the compartment.
(c) Incomplete patterns are obtained from cells in *early* S during the period when zones are starting up. (We expect such patterns from late S when zones are closing down, irrespective of BrdU duration.)
(d) Late-replicating heterochromatin may be present or absent in adjacent metaphases and progressive stages of late X replication are seen.

If BrdU incorporation continued for a long time after washing out, we would not expect to find these features. We conclude therefore that we really are dealing with a pulse situation, although the actual duration or availability could be a few minutes longer than the exposure time stated.

The unclassifiable cells

Relatively little work on replication at band level has been done using pulse regimes; most workers use terminal protocols. One reason is that the usual TB pale staining methods do not allow detection of short BrdU exposures. Such pulse work as has been done has produced contradictory findings. The majority of authors report that the R- and G-zone patterns are mutually exclusive. One or the other is found and there are almost no intermediate patterns. This is consistent with the idea of a distinct cessation or reduction in synthesis between completion of R-zones and the commencement of G-zones somewhere in mid-S (Schempp and Vogel 1978, 1979; Schmidt 1980).

Conversely, others report that intermediate, mixed R/G zone patterns are found at certain stages of S (Dutrillaux 1975) which, whilst not inconsistent with a mid-S reduction in DNA synthesis, does rule out the idea of a clean partition between R- and G-zones and complete cessation of DNA replication at the cell level.

Our findings are clearly consistent with the results of Dutrillaux (1975). We believe that the unclassifiable cells are not a technical artefact of short pulses, nor a reflection of reduced or inefficient BrdU uptake, but represent a real stage in the replication programme for the following reasons:
(a) They do not occur throughout S-phase, but are confined to a specific period in early/mid-S. Here the cells behave as a distinct cohort, with a rise and fall in frequency just as is found in other, more objectively definable stages.
(b) Unclassifiable *regions* occur within cells having clear R- or G-zone patterns as well (impure cells).
(c) Although extension of pulse duration reduces their frequency and shifts the residue to an earlier position in S (Table 1), they are not eliminated, even with a pulse of 60 min, and that is an excessively long time for a pause in an S-phase of ~ 6 h duration.
(d) Longer pulses towards the middle of S produce a very low frequency of mixed pattern cells, which we might call "unambiguous intermediate"; all three zone patterns occur within one cell.
(e) Close inspection of unclassifiable cells often shows, just as in the 5 min pulse situation, that the bands are fragmented, hence the reticulate and confused patterns which are not readily classifiable against a standard banding diagram.

All these things are consistent with a transition stage in replication. Perhaps one is seeing the rapid migration of replication wave fronts between the starting R-zones and the finishing G-zones such that for a (short) time replication activity may be found in both. However it dies out in the R-zones and replication is confined thereafter exclusively to the G-zones (and later constitutive and facultative heterochromatin) for the remainder of S-phase.

The impure patterns are readily explicable when we take into account the very staggered R-zone start at the commencement of S-phase. As a consequence of this, the earliest zones to start will reach transition whilst the activity of others is confined within a zone and still other bands within the karyotype have not even started. The reverse situation will obtain at the end of transition.

We have also to remember that the zones (bands) defined are all compounded from many smaller bands (Bigger and Savage 1975, Yunis 1981), and a whole complex programme of replication is going on at this lower level, readily observable in prophase, which has not yet been mapped (Savage et al. 1984). It is hardly surprising,

therefore, if the shorter pulses begin to reveal this programme discontinuity. Even so, in molecular terms our resolution is very poor: an average chromatid band at the ISCN (1981) 400 band level contains about 7×10^7 bp (Lima de Faria 1983).

We envisage that, by lengthening the pulse, the sub-pattern seen in early S is blurred, and more of the wave-front movement is recorded. Consequently the TB bands become larger and more solid, allowing the eye to observe the expected discrete pattern.

The 3C pause

The postulated quiescent "valley" between R-zone and G-zone replication has been termed the 3C pause (for review see Holmquist et al. 1982). We pointed out above that whilst an intermediate pattern is not inconsistent with a reduction in "rate" of synthesis within S, our findings do not support the idea of a clean pause at the R–G zone junction. Certainly, the existence of mixed pattern cells would militate against this.

Furthermore, a complete cessation would cause the S-cells involved to be devoid of bands with a pulse whose duration is short compared with the pause. This leads to the expectation of a rise in pale cells in the mid-regions of S. Such a rise is completely missing at all pulse durations tested (Fig. 3, Table 1). We are led to the conclusion that replication is a more or less continuous process, operating at all stages of S. A similar conclusion was drawn from a thymidine pulse experiment by Cawood and Savage (1985) using Syrian hamster cells.

The clarity and intensity of G-zone patterns is certainly much more marked than those for R-zones at all pulse durations. Whilst this could reflect fewer replicons operating or that they are more dispersed in R-zones (Hand 1978; Holmquist et al. 1982) other explanations are possible. One is that the staggered start, coupled with a rapid transit to the transition phase (note in Fig. 3 that R-zone and unclassifiable cells occupy a similar position indicating close proximity in time) does not permit the build-up of a clear and distinctive R-zone pattern. Another is that the R-zone sub-programme at mini-band level may be much more complex and the band may appear much less compact when pulse picks up the more scattered activity. Still another is that G-zones are known to be A-T rich (Holmquist et al. 1982) and hence might be expected to have a greater uptake of BrdU for a given pulse duration.

Pulse protocol and sub-phasing

The ability to sub-divide S into defined chronological segments (sub-phases) by objective criteria using replication bands has proved very useful experimentally in a number of areas, particularly in kinetically perturbed situations (Savage et al. 1983; Savage and Cao 1985; Savage and Reddy 1987; Savage and Prasad 1988).

So far, only terminal protocols have been used. RHQ staining now opens up the possibility of using pulse regimes. However, the dependence of cell types and patterns observed upon the duration of the pulse, makes the selection of suitable criteria rather difficult. For example, for pulses as short as 10 min, a crisp G-zone pattern in later S cells is always obtained, but Table 1 shows that longer pulses are required in order to get a clearer picture of early and mid-S. A search for a cell classification that is independent of pulse duration is part of our ongoing investigations.

The work reported here is for stimulated lymphocytes. We have applied the technique also to fibroblasts where a very similar picture emerges. All the cell types recorded here are found there also and we have been able to do some more detailed quantitative work on the phase progressions which we will report at a later date.

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