Cytological subdivision of the S phase of human cells in asynchronous culture

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SUMMARY A method is described for subdividing S phase cells in asynchronous cell cultures on the basis of replication band patterns produced in chromosomes by bromodeoxyuridine incorporation. The criteria used for cell classification are objective, requiring the presence or absence of specific bands on particular chromosomes, and therefore lead to subdivisions amenable to quantitative analysis and for comparative purposes. Two schemes are given: key 1, based on bands in chromosomes 2 and 5, leads to five sub-phases; and key 4, based on bands in chromosomes 3 and 4, leads to four sub-phases. The order of the sub-phases, though not their relative durations, is identical in the six primary cell cultures (four fibroblast and two lymphocyte) tested. The technique provides for a detailed study of the programme of chromosome replication in normal and abnormal cells which, in time, should produce new criteria for diagnostic purposes.

For several years, we have had available a system, applicable to exponentially growing asynchronous cultures of Syrian hamster cells, which allows us to subdivide S phase cells on the basis of their replication band patterns produced by bromodeoxyuridine (BrdU) incorporation. The method is not based upon generalised classification, but is objective, requiring the presence or absence of specific bands on particular autosomes.^{1,2}

In addition to the traditional analysis of the cell cycle, we can 'unscramble' S phase, and replace cells in correct chronological sequence and so monitor normal and experimental changes as a cell transits this compartment.^{3,4}

We can also isolate, cytologically, a narrow cohort of cells at a precise developmental stage, thus facilitating valid qualitative and quantitative comparison between individuals and experimental treatments free of the background 'noise' inevitably present in time samples from an asynchronous population.⁵⁻⁷

This paper reports the development and initial testing of a similar system, using the same principles, applicable to human cells in culture. It is designed as a practical paper to introduce the method to would-be users, and in accord with this purpose, presentation of detailed results and discussion thereof has been kept to a bare minimum.

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It is shown that a hamster type system is readily applicable to both fibroblasts and lymphocytes, and subdivision of S into five (key 1) or four (key 4) sub-phases is easily and repeatably achieved.

Principle of the method

If BrdU (10 to 20 µg/ml) is added to a culture of cells in exponential growth, all subsequent DNA synthesis will incorporate some brominated uridine as a substitute for thymine. Consequently, cells with chromosomes that were in the process of replication will arrive at metaphase with various mixtures of substituted (TB) and unsubstituted (TT) chromatin, the latter being the chromatin that had completed replication before the BrdU arrived. Careful staining by the Hoechst/light/Giemsa method^{2,8} causes such chromosomes to display patterns of dark staining (TT) and light staining (TB) bands, the number and arrangement of which will depend upon where the cell was in its synthesis programme when BrdU was added.

If cultures are serially sampled at suitable intervals after addition of BrdU, the rise and fall in the fraction of such differentially stained metaphases (FDM) is very similar to the FLM obtained with a pulse of tritiated thymidine, and can be used in exactly the same way to estimate cell cycle parameters.⁹

We now make two further assumptions.

(1) Each pair of homologous autosomes has a fixed programme of replication, bands and regions

completing in a regular sequence which is the same for each cell in a particular population.

(2) Although each chromosome follows its own programme with an element of independence, within a cell the various members of the complement keep in fairly close step with one another. This means that given the pattern stage reached by one member, that displayed by other members can be predicted with reasonable accuracy.

These assumptions have proved to be valid for Syrian hamster cells, and so far appear to hold for human cells also.

Fig 1 illustrates the principle of S phase subdivision based on these assumptions. Given a band on an easily recognised autosome that makes its appearance (that is, it has, before the BrdU arrives, replicated sufficient TT DNA to produce a discrete dark band) part way through S, then all S cells arriving at metaphase can be classified into two groups, those with the band (B, fig 1) and those without (A), the former being chronologically later in S than the latter. Provided that the time of appearance is well defined, a plot of the frequency of the two types with sampling time will produce a steep curve (the 'band appearance curve') from which one may determine the average position in S where the band completes replication.

If we now consider a second band which completes at a different time in S (C, D, fig 1) then a consideration of the status of both these bands within a cell will produce three classes of S cell, BD, BC, and AC, and the relative frequency of these classes with sampling time will indicate their chronology. Further bands can be added to produce four, five . . . subdivisions.

In practice, we have found four or five subdivisions can be achieved easily and regularly. Provided the relative sub-phase frequencies for a complete S wave are obtained accurately, some indication is given of cell mixing within the population, and very reasonable estimates can be made of the average durations and border positions of sub-phases.

Selection of suitable bands

Preliminary work was done with untransformed human fibroblasts of fetal lung origin, line HF19, which has been used extensively for experimental work in this Unit. Batches were brought up from liquid nitrogen storage and cultured in Eagle's MEM+10% calf serum, L glutamine, and antibiotics. BrdU at final concentration 20 μ g/ml was added to exponentially growing cultures in Falcon

FIG 1 The principle of S phase subdivision. Two chromosomes are shown with particular bands 'completing' replication at different times during S. With serial sampling, a plot of the relative frequencies of the two classes of cells (A, B or C, D) with time produces a 'band appearance' curve (which may be plotted in two ways, see A, B, above) from which an estimate of the time of completion of a band may be derived. If these bands are used in combination to delimit borders, S may be divided into three chronological sub-phases whose distribution in time may be plotted and durations computed.

flasks, and replicate pairs of flasks were sampled every 2 hours up to 16 hours (each with a 2 hour colcemid pretreatment). Air dried metaphase preparations were stained for replication bands using the Hoechst/light/Giemsa method of Cawood.²

An initial survey of band frequency histograms for chromosomes 1 to 5 led to a selection of 16 likely bands. Further analysis (taking into account such factors as ease of recognition in cells of different quality, homologue disparity, steepness and position of the time of appearance curve, etc) led to the selection of 10 criteria for rigorous testing. These are shown in fig 2, superimposed on R band patterns derived from ISCN (1978).¹⁰

Several keys for subdivision of S phase were constructed from numerical scores of these criteria, and the two deemed most suitable for further development are given in the next section. The arrangement is a series of mutually exclusive questions just like the 'artificial keys' found in Floras. Band numbering is according to ISCN (1981).¹¹

The subdivision keys

KEY 1

This is based on criteria from chromosomes 2 and 5 and results in five sub-phases, numbered (following the precedent of Syrian hamsters) Sk1-5.

α	{	At least one 2q devoid of replication bands	Sk1
	{	Obvious bands on both 2q	
β	{	2p with distinct mid-gap (region 14 to 16)	γ
	{	2p mid-gap absent (or very poorly demarcated)	δ

R BAND PATTERNS AFTER ISCN (1978)

γ	{	5q with distinct terminal gap (34).....	Sk2
	{	5q without terminal gap	Sk3
δ	{	5p with distinct mid-gap (14).....	Sk4
	{	5p solid, without distinct, clearly demarcated gap	Sk5

Most criteria concern the in-fill of defined gaps ($\equiv$ dark G bands). The first question in each step is positive, a 'yes' answer for either homologue being required. The 5q34 gap (γ) can be difficult in cells with over-contracted chromosomes as the three dark R bands of region q3 tend to fuse. The proximal bands 2q11 are usually the first to appear in the long arm.

KEY 4

This is based on criteria from chromosomes 3 and 4 and results in four sub-phases SkI-IV. Roman numbers are used to avoid confusion with subdivisions of key 1.

α	{	At least one 4q without obvious proximal replication band in position (21)	SkI
	{	Both 4q with obvious (21) proximal bands	β
β	{	3p with clear proximal gap (11 to 12).....	SkII
	{	3p without clear proximal gap.....	γ
γ	{	4q with clear, well demarcated proximal gap (13)	SkIII
	{	4q without obvious proximal gap	SkIV

Most criteria concern the in-fill of defined gaps ($\equiv$ dark G bands). Chromosomes 4 and 5 can be confused in very early S when few bands are about. Chromosome 5q nearly always displays the sub-median band (31) either before or contemporary with

FIG 2 The final selection of 10 criteria (mostly pale R bands $\equiv$ dark G bands) tested in human fibroblasts (strain HF19) for suitability as sub-phase border delimiters. Seven of these were used to construct two keys suitable for subdivision of S.

any proximal bands. Chromosome 4 nearly always displays the proximal (21) first and any other band is small and central (25 to 27) rather than sub-median.

Comments on the use of the keys

THE EARLIEST STAGE OF S (Sk1, SkI)

A cell is defined as being in S if, and only if, one or more distinct red stained bands are present within an arm or arms in an intercalary or non-centric position.

In this we differ from some others^{12,13} who count staining (often asymmetrical) of the paracentric heterochromatin of 1, 9, and 16 as the first stage. We believe this to be only a relative darkening of those regions, for at later sub-phases (Sk3 to 5, III to IV) they appear as distinct pale staining gaps, and remain so until almost the end of S. Similar transitory centromeric staining occurs in Syrian hamsters.² Such disappearance of replicated bands is precluded by the nature of the BrdU protocol; dark staining bands are predominantly TT and do not take up (or take up only minimal amounts of) BrdU before reaching metaphase.

The first bands to appear are not always on the same chromosome, but chromosomes 1 and 3 and the E group, particularly 17, are usually among the earliest.

A problem that can lead to confusion in very early S cells particularly if over-staining is present is that of 'shadow bands', the chromosomes displaying an almost complete set of R bands. Without care, when such 'shadow bands' are present, an early cell can easily be classified as a later sub-phase. A yellow or yellow-green filter helps here, for true (replicated) bands show up as discrete reddish-brown coloured dots while unreplicated chromatin and 'shadow bands' remain blue-grey. Phase contrast can also be used to distinguish between replication and 'shadow bands'. Nevertheless, it is necessary to be ruthless in scoring, accepting only those bands where doubt does not exist. These 'shadow bands', of which the paracentric heterochromatin discussed above may be a sub-set, probably represent subtle structural or condensation differences which exist along the arm at this stage, but they may also indicate incipient replication, a case where insufficient TT chromatin has been generated to produce a discrete band, but sufficient to induce a generalised darkening. This we have observed in Syrian hamsters and know from combined replication banding/³H thymidine studies that some replication occurs before a cell is classifiable as being in S by BrdU staining. If care is taken with staining, 'shadow bands' do not present major problems.

HOMOLOGUE DISPARITY

Although all autosomes within a cell tend to keep in

step (assumption 2), an element of independency does lead to slight differences in the time of band appearance between homologues.¹⁴ This is not because one homologue is always in advance of the other since individual bands within the pair may differ in opposite directions. For a fuller discussion see Savage *et al.*⁷

Such disparity, which usually amounts to only a few percent of pairs examined, can lead to difficulties in cell classification when it concerns the specific key bands. For this reason it has become necessary to establish certain rules to ensure consistency between scorers. We have chosen the rule of the 'positive question': if the answer to the first question in each section of a key is unequivocally yes for either homologue, the answer is yes for that cell.

Thus, cell classification is ultimately based on one homologue, and this has a two-fold advantage. First, it is frequently the case that only one homologue is readily visible and analysable and, secondly, for many cell kinetic studies, a cell does not have to be complete to be usable.

THE TERMINATION OF S

The human eye is much better at discriminating dark objects than pale ones, and it would have been better to have built the keys using the appearance, with time, of specific, isolated dark bands. This has not proved possible in human cells. The three dark bands tested (4q21, 4q25 to 27, 5q31; fig 2) all have band appearance curves which are superimposable, so only one was usable. In general, the early replicating dark R bands of chromosomes 2 to 5 show less variation in time of appearance than the later replicating pale R bands ($\equiv$ dark G bands). Consequently, in order to secure good spacing of appearance curves throughout S, most of the criteria are of the gap closure type (C, D; fig 1).

As the chromosomes come to the very end of S, only very small scattered gaps are left, and these can easily be overlooked. The difficulty is exacerbated because the SSC treatment necessary to elicit the staining reaction tends to produce coils in post-S chromosomes giving them a banded appearance. Careful observation will indicate that this is a typical G band pattern, but it is not always obvious.

In practice, the paracentric heterochromatin of 1, 9, and 15p are the last obvious 'gaps' to disappear and can safely be used as termination criteria, even in the presence of incipient coiling.

A similar problem exists in Syrian hamsters, and we have investigated it using combined tritiated-thymidine pulse autoradiography and BrdU replication banding in the same cells and have shown that the differences between the posterior border of S determined from the FLM and FDM are quite small.⁹

Results

HF19 FIBROBLASTS

Fig 3 shows the 'band appearance' curves for the criteria used in the two keys. There are several ways to plot such data; here, the ordinate is the frequency of S cells in each time sample with a clear gap in at least one homologue. It will be seen that the curves are sigmoidal and quite steep (indicating that assumption 2 is being fulfilled) and well spaced out through S.

Fig 4 shows frequency polygons of the actual subdivisions obtained. The extensive overlap serves to emphasise the considerable mixture of chronologically different developmental stages which exist as a norm in asynchronous populations, and cautions against placing too much trust in time sampling for quantitative work in such populations.

FRESH FIBROBLAST EXPLANTS

Having established that S phase subdivision is possible with HF19 human fibroblasts in asynchronous culture, it is necessary to test the system on

FIG 3 The 'band appearance' curves in HF19 fibroblasts for bands used to delimit borders for key 1 (upper set) and key 4 (lower set). As 2 hour colcemid accumulations were used, frequencies are plotted to the mid-point of the time periods. Compare fig 1 and note that the curves are steep and well separated.

FIG 4 Sub-phases in HF19 produced by the two keys. The terminology 'Sk' follows the precedent set in hamsters and indicates the use of a terminal BrdU pulse protocol.¹ Key 4 uses Roman numerals to avoid confusion with the sub-phases of key 1. Note the chronological order of the frequency polygons and also the heterogeneous mixture of cells of different ages which occurs in each metaphase sample.

other batches to check its repeatability and the range of variation one might encounter in 'field' conditions. It is also desirable to test other cell types, for example, blood lymphocytes as well as cultural variations of growth media, serum source, etc.

If, for the key bands used, a consistent replication order is maintained, regular and comparable subdivision becomes possible, and the system may be applied to the study of abnormal cells and for experimental purposes. Such consistency has been amply confirmed for Syrian hamster cells and there the method is in routine use for a wide variety of studies.

Three samples were obtained from aborted fetuses and short term primary fibroblast cultures established in Eagle's MEM with 10% fetal calf serum. At the third passage, cells were seeded into Falcon flasks and allowed 30 hours to establish exponential growth. Then BrdU was added and

KEY 1**KEY 4**

FIG 5 Subdivision produced by the keys in three primary fibroblast cultures. HF1:46,XX, normal. HF2:46,XY, satellite on Yq. HF5:46,XX;t(3;22) (p11;q11). The order of sub-phases and borders is the same in all samples and identical to that found in HF19. The average border positions and therefore average sub-phase durations differ between samples (see insets which give relative durations) and the variation is not simply related to differences in S phase duration (SDUR).

serial samples taken every 2 hours (with 2 hour colcemid accumulations) up to 16 hours (only 11 hours were attained with HF1 owing to shortage of material). Air dried metaphase preparations were stained for replication bands and coded, randomised slides scored for sub-phases using the two keys given above.

HF1: 46,XX, chromosomally normal: established from fetal skin.

HF2: 46,XY, with a satellited Yq. The origin of the satellite has not been determined: established from fetal skin.

HF5: 46,XX,t(3;22)(p11;q11): established from fetal diaphragm.

No major difficulties were encountered in applying the keys.

Fig 5 summarises the results. The order of the sub-phases and of the band appearance curves is identical in every sample, and is the same as that established for HF19. This is true for the keys considered separately, and for the order of the sub-phase borders for both keys combined.

Differences in the average durations of particular sub-phases occur between the samples (that is,

FIG 6 Subdivision in stimulated normal blood lymphocytes from a 52 year old male and a 23 year old female after 46 hours in culture. Average G_2 and S duration (SDUR) are shorter than in fibroblasts. The order of borders and sub-phases is identical to that in fibroblasts (that is, the replication order of the key bands is constant for all samples tested to date) but spacing differs. However, border differences between the two blood samples are not (key 1) or only marginally (key 4) significant.

border spacing or average time of band appearance differs), and these do not appear to be directly proportional to the differences in overall S duration recorded (the 'stretching' of S is non-elastic). The samples are too few as yet to investigate this phenomenon more fully.

CIRCULATING BLOOD LYMPHOCYTES

Two cultures of normal blood lymphocytes (one from a 52 year old male, the other from a 23 year old female) have been tested, the BrdU (10 µg/ml) being added about 46 hours after stimulation followed by 2 hour serial samples. Fig 6 shows that average S duration was shorter than in fibroblasts so that sub-phase borders are closer in absolute time. Nevertheless, the order of all sub-phase borders from the use of keys 1 and 4 was identical to that obtained in fibroblasts. The border positions and relative sub-phase durations differed from fibroblasts, but not (key 1) or only marginally (key 4) between the two lymphocyte samples.

Discussion

Since this paper is primarily intended as a practical report, results have not been given in detail and therefore in-depth discussion must be deferred.

There have been many experimental investigations of replication in human cells using BrdU incorporation,^{8 14-18} and although most have attempted to determine the order of band replication within individual chromosomes, few have attempted to subdivide S to obtain estimates of the relative times of replication of bands or durations of the chronological steps suggested. Recently, Camargo and Cervenka¹³ used a five-fold subdivision in lymphocytes following release from a methotrexate block. The perturbed, semi-synchronous nature of this population, and the subjective subdivision criteria used, preclude direct quantitative comparison with our subdivisions.

The tests reported here on both normal and chromosomally abnormal cells show a consistent order of appearance of the key bands (and hence of the sub-phases) as cells transit S, and this is an encouraging indicator that the system will prove as usable and versatile as it has in hamsters.

The variations of average sub-phase durations (and relative border positions) between samples is larger than we have experienced in the hamster. In part, this may reflect the much greater genetic diversity which exists between humans compared with inbred laboratory rodents. However, the number of samples examined so far is small, and two at least were chromosomally abnormal. If the variation, however, proves to be really large and

independent of the vagaries of culture conditions (a factor not yet investigated), this may limit the usefulness of the scheme for comparative purposes, in particular the investigation, perhaps even diagnosis, of certain abnormal syndromes.

It must be recognised that the subdivision we employ is utilitarian and arbitrary in the sense that it is not consciously related to any cellularly controlled programme of replication. We note, as many others have,^{8 13 14 19} that replication and 'bands' are related and that the earliest bands to replicate correspond to dark R bands. Whether a sharp break occurs between dark R and dark G, or whether discontinuities or rest periods occur at various stages, are points that our subdivisions do not decide; the sub-phase borders were not chosen to correspond with such events, but to provide division of S phase into four or five more or less equal time segments.

The chief advantage of the method is that it gives some degree of independency of sampling time with asynchronous populations, and by allowing S phase to be unscrambled permits reliable qualitative and quantitative changes to be monitored as a cell transits S.

Furthermore, a narrow cohort of cells at a precise developmental stage can be isolated cytologically, and valid quantitative comparisons made between cultures, tissues, and experimental treatments (and, hopefully, individuals) without the background noise inevitably present in a time sample from such populations. With such cohorts, the replication programme for individual chromosomes, and parts of chromosomes, can be studied and compared with considerable precision^{5 7} and this should allow the regular detection of anomalies, some of which could have diagnostic value.

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References

- 1 Savage JRK, Bhunya SP. Cytological sub-division of S-phase in the Syrian hamster (*Mesocricetus auratus*). *Chromosoma* 1980;77:169-80.
- 2 Cawood AH. Chromosome replication in fibroblasts of the Syrian hamster (*Mesocricetus auratus*). *Chromosoma* 1981;83:711-20.
- 3 Savage JRK, Bhunya SP. The induction of chromosomal aberrations by X-irradiation during S-phase in cultured diploid Syrian hamster fibroblasts. *Mutat Res* 1980;73:291-306.
- 4 Savage JRK, Breckon G. Differential effects of sulphur mustard on S-phase cells of primary fibroblast cultures from Syrian hamsters. *Mutat Res* 1981;84:375-87.

- ⁵ Savage JRK, Cawood AH, Breckon G. Early-replication DNA patterns in a derivative chromosome in a Syrian hamster with only 42 chromosomes. *Chromosoma* 1982; **84**:505-9.
- ⁶ Savage JRK, Papworth DG. The effect of variable G₂ duration upon the interpretation of yield-time curves of radiation-induced chromatid aberrations. *J Theor Biol* 1973; **38**:17-38.
- ⁷ Savage JRK, Cawood AH, Papworth DG. The disparity between homologous chromosomes during DNA replication. *J Theor Biol* 1983; **100**:631-43.
- ⁸ Kim MA, Johannsmann R, Grzeschik KH. Giemsa staining of the sites replicating DNA early in human lymphocyte chromosomes. *Cytogenet Cell Genet* 1975; **15**:363-71.
- ⁹ Cawood AH, Savage JRK. A comparison of the use of bromodeoxyuridine and [³H] thymidine in studies of the cell cycle. *Cell Tissue Kinet* 1983; **16**:51-7.
- ¹⁰ ISCN (1978). An international system for cytogenetic nomenclature. Report of the standing committee on human cytogenetic nomenclature. *Cytogenet Cell Genet* 1978; **21**:309-404.
- ¹¹ ISCN (1981). An international system for human cytogenetic nomenclature—high resolution banding. *Cytogenet Cell Genet* 1981; **31**:1-32.
- ¹² Camargo M, Cervenka J. Pattern of chromosomal replication in synchronized lymphocytes. I. Evaluation and application of methotrexate block. *Hum Genet* 1980; **54**:47-53.
- ¹³ Camargo M, Cervenka J. Patterns of DNA replication of human chromosomes. II. Replication map and replication model. *Am J Hum Genet* 1982; **34**:757-80.
- ¹⁴ Dutrillaux B, Couturier J, Richer CL, Viegas-Péquignot E. Sequence of replication in 277 R- and Q-bands of human chromosomes using a BrdU treatment. *Chromosoma* 1976; **58**:51-61.
- ¹⁵ Dutrillaux B, Laurent C, Couturier J, Lejeune J. Coloration par l'acridine orange de chromosomes préalablement traités par le 5-bromodeoxyuridine (BUDR). *CR Acad Sci [D] (Paris)* 1973; **276**:3179-81.
- ¹⁶ Epplen JT, Siebers JW, Vogel W. DNA replication patterns of the human chromosomes from fibroblast and amniotic fluid cells revealed by a Giemsa staining technique. *Cytogenet Cell Genet* 1975; **15**:177-85.
- ¹⁷ Grzeschik KH, Kim MA, Johannsmann R. Late replication bands of human chromosomes demonstrated by fluorochrome and Giemsa staining. *Humangenetik* 1975; **29**:41-59.
- ¹⁸ Kondra PM, Ray M. Analysis of DNA replication patterns of human fibroblast chromosomes. *Hum Genet* 1978; **43**:139-49.
- ¹⁹ Ganner E, Evans HJ. The relationship between patterns of DNA replication and of quinacrine fluorescence in the human chromosome complement. *Chromosoma* 1971; **35**:326-41.

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