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Correspondence and requests for materials should be addressed to R.S.M. (e-mail: molday@unixg.ubc.ca). or K.-W.Y. (e-mail: kyau1@jhem.jhmi.edu).

Establishment of transcriptional competence in early and late S phase

Jianmin Zhang*†, Feng Xu*†‡, Tamar Hashimshony*, Ilana Keshet* & Howard Cedar*

* *Department of Cellular Biochemistry and Human Genetics, Hebrew University, Ein Kerem, Jerusalem, Israel*
† *These authors contributed equally to this work*

In animal cells, the process of DNA replication takes place in a programmed manner, with each gene region designated to replicate at a fixed time slot in S phase. Housekeeping genes undergo replication in the first half of S phase in all cell types, whereas the replication of many tissue specific genes is developmentally controlled, being late in most tissues but early in the tissue of expression[1]. Here we employ nuclear DNA injection as an experimental system to test whether this phenomenon is due to differences in the ability to set up transcriptional competence during S phase[2,3]. Our results show that, regardless of sequence, exogenous genes are a better template for transcription when injected into nuclei of cells in early as opposed to late S phase, and this expression state, once initiated, is preserved after cell division. DNA injected in late S phase is apparently repressed because it is packaged into chromatin containing deacetylated histones, and the same is true for late replicating chromosomal DNA. These findings suggest a mechanistic connection between replication timing and gene expression that might help to explain how epigenetic states can be maintained *in vivo*.

Non-synchronized Rat-1 cells were plated onto a marked gridded disk at a density permitting the identification of each individual cell by its position on the plate. Cells were randomly injected with an S16–LacZ reporter plasmid and each one was followed by microscopy at 2-hour intervals to determine its time of division and thereby ascertain its cell cycle position at the time of injection (Fig. 1a). At 20 h after injection, cells were fixed and stained for LacZ activity and the number of positive cells was graphed as a function of cell cycle stage at the time of injection.

S16–LacZ gene sequences injected into early-S-phase cells were more efficiently transcribed (~10-fold) than when injected into late-S-phase cells (Fig. 1b). Regardless of the stage of the cell cycle at which the injection took place, activity was always determined after 20 h, so that in each case the cells went through a full cycle and had the same amount of time to accumulate product. Another expression vector made up of the EF1-α housekeeping promoter linked to a green-fluorescent-protein (GFP) reporter showed a similar difference between injection during early and late S phase (data not shown), and the same was true for tissue specific promoters such as α-actin or myogenin (Fig. 1b), indicating that this is a general, sequence-independent phenomenon. Expression competence, once established, seems to remain fixed even though the cell continues to go through its normal division cycle. Thus, late-injected cells retain a low transcription rate even 48 h after injection, whereas the early-injected substrate maintains its high level of expression (Table 1).

The fact that templates injected into late-S-phase cells remain relatively inactive, even after many hours in the cell, strongly suggested that transcriptional competence is established and fixed soon after injection. To prove this directly, we designed a new injection protocol (Fig. 2a) that allowed us to isolate early-injected and late-injected cells and assay transcription directly by polymerase chain reaction with reverse transcription (RT–PCR) (Fig. 2b). After 3 h, early-S-injected S16-β-globin was already more transcriptionally efficient (6.7-fold) than a template injected during late S

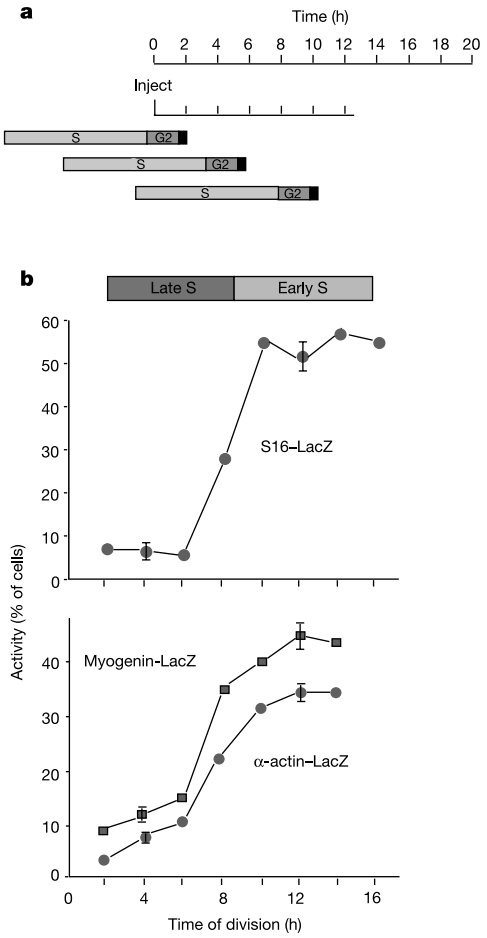


Figure 1 Transcriptional competence as a function of S phase. **a**, Cells on a Cellocate grid were injected (long vertical bar) at zero time and followed every 2 h (short bars) to determine whether mitosis (black) had occurred. Mitosis at 10 h (bottom cell) indicates that injection took place in early S. **b**, Rat-1 cells were injected with S16–LacZ (0.75 ng μl^{-1}; 700 cells), myogenin–LacZ (1.25 ng μl^{-1}; 850 cells) or α-actin–LacZ (0.8 ng μl^{-1}; 1,250 cells) and assayed for LacZ-positive cells after 20 h. Error bars indicate the 95% confidence interval for the 2-h, 4-h and 6-h or the 10-h, 12-h and 14-h points as a group. The diagram at the top indicates cells injected at early S (dark grey) or late S (light grey).

‡ Present address: Toronto General Hospital, 200 Elizabeth Street, MBRC 1-934, Toronto, Canada ONT M5G 2C4.

phase, and similar results were obtained even after 1 h (data not shown). These differences were not simply due to effects of the cell cycle on template stability, because exogenous plasmids were present in equal amounts whether injected early or late in S phase (Fig. 2c).

As a final test of the idea that it is indeed the time of injection that determines transcriptional competence, we designed an experiment to measure the expression of early S and late S templates in a single cell. Whereas cells injected with the S16–LacZ reporter construct in early S were very efficient in initiating transcription (63%), only 19% of this same cell population expressed an E1F-α–GFP plasmid injected in late S (Fig. 2d). Similar results were obtained when the GFP reporter was injected in early S and the S16–LacZ in late S, or even when the protocol was reorganized so that the first injection was directed into cells in late S. These results clearly indicate that DNA initially exposed to late S remains relatively inactive, even though new vectors injected into early S are highly transcribed in the same cells.

We next examined the chromatin structure of injected DNA. Using quantitative PCR analysis we first showed that exogenous templates are present in nucleosomal DNA at about the same level as endogenous sequences (β-actin), in both early-injected and late-injected cells (Fig. 3a).

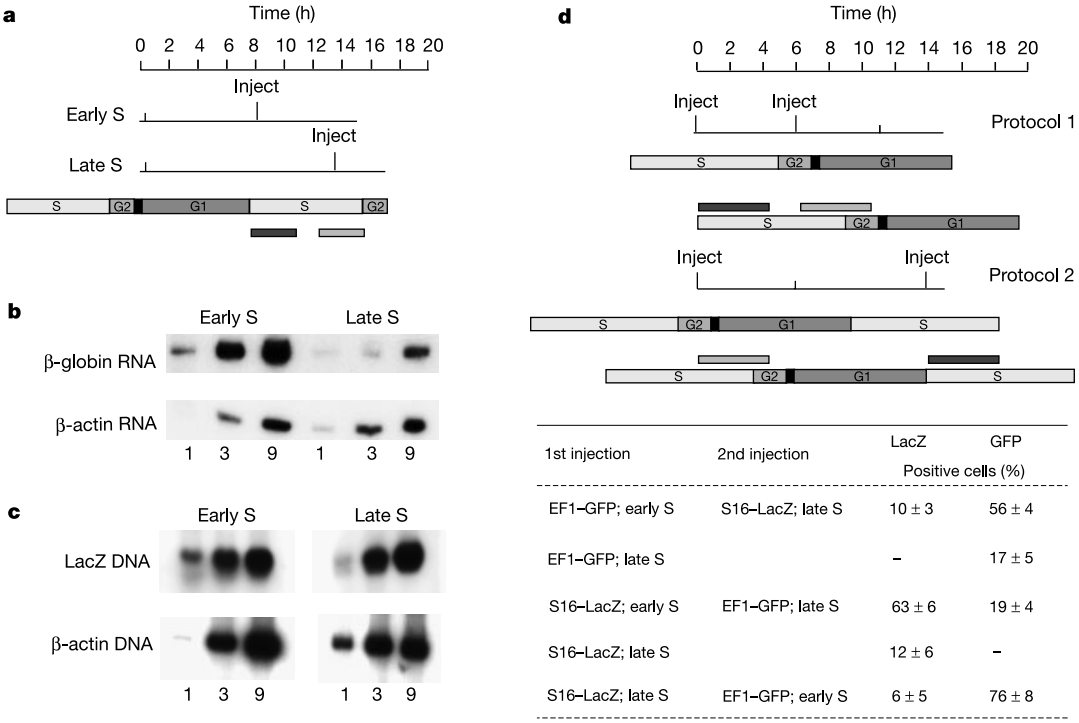
We then characterized histone acetylation[4,5] by employing two plasmids that differed from each other by a small deletion (Fig. 3b). One plasmid (I) was injected into cells in early S, and the other (II) was injected in late S (exp. 1). Nucleosomes from the combined cell populations were immunoprecipitated with anti-Ac-H4 and assayed by quantitative PCR, using β-actin and Ig-κ as active (enriched) and inactive endogenous gene controls. Early-injected DNA was about 10-fold more enriched for acetylated histones than late-injected DNA (Fig. 3b and d). Similar results were obtained in a reciprocal injection experiment (exp. 2) or when S16–LacZ was used as the template (see legend to Fig. 3). These findings indicate that DNA injected in early and late S adopt different chromatin structures and that this is not dependent on the presence of an active promoter.

These studies indicated that the repression of late-S-injected reporter genes might be due to the presence of deacetylated histones. To test this hypothesis, we injected the S16–LacZ reporter gene into cells in early or late S that had been pretreated with a short pulse of trichostatin A (TSA). This treatment led to a striking increase (2.8-fold) in expression of late-S-injected DNA (Fig. 4a), indicating that histone deacetylation partly accounts for the repression phenomenon. However, once this state is set up in late S it remains stable and even subsequent exposure to TSA several hours

Table 1 **Transcriptional competence as a function of S phase**

Gene	Assay time (h)	LacZ-positive cells (%)	
		Injection in early S	Injection in late S
S16–LacZ	24	69 ± 8	22 ± 8
	48	65 ± 9	17 ± 6
Myogenin–LacZ	24	46 ± 10	14 ± 6
	48	43 ± 10	9 ± 4

Rat-1 cells were injected with S16–LacZ (0.75 ng μl^{-1}) or myogenin–LacZ (1.25 ng μl^{-1}) and assayed for LacZ-positive cells after 24 or 48 h; means ± 95% confidence interval are shown. Cells that divided within the first 6 h after injection were considered late-S recipients, whereas those that divided within the next 6 h were considered early-S recipients.



1st injection	2nd injection	LacZ	GFP
		Positive cells (%)	
EF1–GFP; early S	S16–LacZ; late S	10 ± 3	56 ± 4
EF1–GFP; late S		–	17 ± 5
S16–LacZ; early S	EF1–GFP; late S	63 ± 6	19 ± 4
S16–LacZ; late S		12 ± 6	–
S16–LacZ; late S	EF1–GFP; early S	6 ± 5	76 ± 8

Figure 2 Characterization of injected DNA. **a**, Recently divided cells (within 3 h) were identified (short vertical bar) by their morphology, and their positions recorded. Injection (long bar) was performed after either 8 h (early S cells) or 13 h (late S cells). Early (dark-shaded) and late (light-shaded) injected time intervals are about 3 h long. **b**, Cells were injected with S16-β-globin (2 ng μl^{-1}) in early S (320 cells) or late S (250 cells) and harvested after 3 h. Quantitative RT–PCR (1, 3 and 9 μl) was performed after adjusting for injected cell number. **c**, S16–LacZ was injected in late S or early S (equal numbers) and assayed for DNA after 3 h. Similar results were obtained for an S16-β-globin template 10 or 24 h after injection. **d**, Rat-1 cells were injected at zero time (early S) with either S16–LacZ (0.75 ng μl^{-1}) or EF1-α–GFP (0.4 ng μl^{-1}). At 6 h, non-divided cells were injected for a second time (late S) and cells that divided within the next 5 h were recorded (protocol 1). The injection time spans for early S (dark-shaded area) and late S (light-shaded area) are shown. In protocol 2, cells were injected at zero time (late S) and those that divided within 6 h were marked and then injected 8 h later (14 h on the time scale) (early S). Results (±95% CI) from 250–380 divided cells for each point are tabulated.

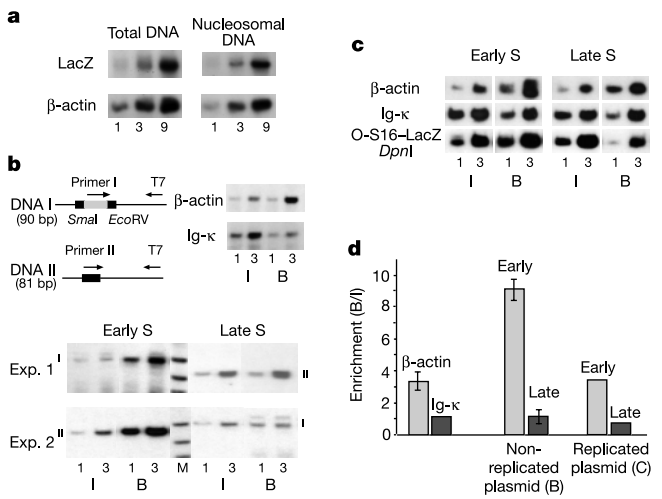


Figure 3 Cell-cycle-dependent histone acetylation. **a**, Nuclei (500) from a tissue culture plate containing 10^5 cells were injected with S16–LacZ, isolated after 24 h and used to prepare nucleosomal DNA for PCR analysis. The ratios of nucleosomal to nuclear DNA for each sequence were 0.9 (β-actin) and 0.8 (S16–LacZ). Almost identical results were obtained for dinucleosomes and trinucleosomes and for S16–LacZ DNA injected (24 h) in early S (0.8) or late S (0.9). **b**, Histone acetylation on newly packaged DNA was assayed after injection (Fig. 2a) of pBluescript II SK (DNA-I) or the same plasmid containing a small deletion near the T7 primer (DNA-II). DNA-I was injected into early-S cells and DNA-II into late-S cells (exp. 1) or the reverse (exp. 2). At 24 h after injection both cell populations were combined and nucleosomes were immunoprecipitated with anti-Ac-H4 (ref. 27). Total input (I) and bound DNA (B) were analysed[28] by quantitative PCR (two concentrations). **c**, Histone acetylation was assayed after injection of O-S16–LacZ into E1/E2-expressing Rat-1 cells in early or late S. Replicated molecules were assayed by digestion with *Dpn*I before PCR. **d**, Summary of results (means ± s.e.) in **b** and **c** relative to Ig-κ (set at 1.0).

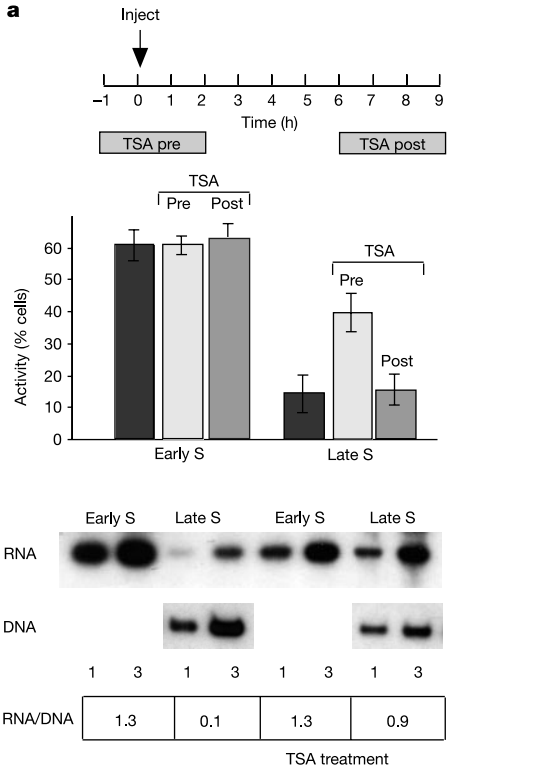


Figure 4 TSA relieves late-S repression. **a**, Rat-1 cells were injected as in Fig. 1 with S16–LacZ (700 cells) or without (800 cells) a 3-h TSA (50 ng ml^{-1}) treatment initiated either 1 h before (pre) or 6 h after (post) injection. Late-S cells were those that divided 2–6 h after injection; early-S injected cells were those dividing 8–12 h after injection. **b**, Cells were injected with S16-β globin as in Fig. 2a and analysed by RT–PCR after normalization to β-actin. TSA was added 1 h before injection. For each experiment, cells injected early and late had similar amounts (by PCR) of DNA (only late is shown).

after injection (post) has no effect. A similar experiment was done by injecting the S16-β-globin reporter and assaying early-injected and late-injected cells by RT–PCR (Fig. 4b). Here, too, TSA treatment overcomes much of the repression observed in late-S-injected cells.

To determine whether differential S-phase nucleosomal packaging also takes place on replicating molecules, we generated a plasmid carrying the bovine papillomavirus (BPV) origin of replication (O-S16–LacZ) and injected it into early or late S-phase Rat-1 cells made replication-competent by previous transfection with an E1/E2-expressing episomal vector[6]. Because the injected DNA is of bacterial origin, it is initially digestible by *Dpn*I (G^mATC) but becomes resistant after replication in animal cells. By this criterion, about 30–40% of the plasmid molecules injected in either early or late S-phase have undergone replication within the first 3 h (data not shown). Mononucleosomes from these cells were immunoprecipitated with anti-Ac-H4 and extracted DNA was then digested with *Dpn*I to eliminate all except the replicated plasmid molecules. Once again, we observed that early-S DNA is assembled into nucleosomes enriched with Ac-H4, whereas late-S-replicated vectors become associated with deacetylated histones (Fig. 3c and d). Similar results were also observed for Ac-H3 (data not shown).

In the light of these results, we then asked whether S-phase control also has a role in setting up the acetylation pattern of nucleosomes assembled on replicating chromosomal DNA. It has already been demonstrated that DNA within late replication bands is specifically associated with deacetylated histone H4, both in general and on the X chromosome in particular[7], but we wished to devise a sequence-independent test of this idea. Rat-1 cells were first stably transfected with a neutral plasmid (pBluescript), and a pool of colonies representing about 200 individual integration events was grown up. We assumed that, in these cells, some insertion sequences replicate in early S, whereas others undergo DNA synthesis in late S, and we wished to determine whether this distribution is correlated with histone acetylation. We therefore performed chromatin immunoprecipitation (ChIP) analysis with anti-Ac-H4 on separate pooled colonies incubated for 1 h in 5-bromodeoxyuridine (BrdU) in either early or late S phase (Fig. 5). Strikingly, early-replicating BrdU-labelled plasmid copies were found to be enriched for acetylated histones, whereas the sequence-identical late-replicating copies in the same cells were depleted. These studies show that, like episomal DNA, chromosomal sequences are specifically packaged into a deacetylated histone structure when replicated in late S.

The original 'window of opportunity' model was based on the concept that cell-cycle variations in transcription factor availability can directly influence the probability of forming stable transcription complexes[2,3]. Our studies support a chromatin-based promoter-independent model whereby DNA becomes packaged differently in early S as opposed to late S. The difference in competence is evidently caused by cell-cycle-dependent factors that influence chromatin structure[8] by altering histone modification[4,9]. One possibility is that, whereas all newly replicated DNA might be initially packaged with Ac-H4 (refs 5, 10), nucleosomes assembled in late S are specifically deacetylated[11]. This is consistent with experiments showing that the histone deacetylase HDAC2 is localized exclusively to late-S replication complexes[12,13].

The process of setting up transcriptional competence most probably represents an integral part of the normal replication

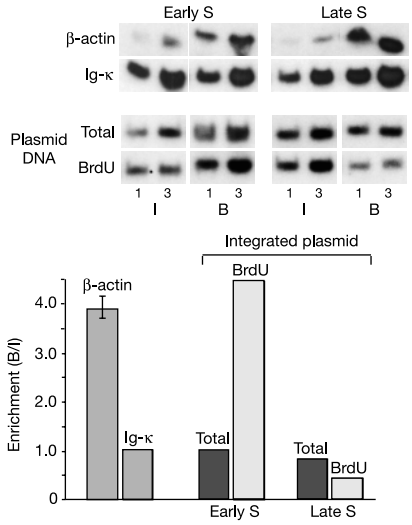


Figure 5 Histone acetylation of early-replicating and late-replicating chromosome DNA. Pools of Rat-1 colonies transfected with the plasmid pBluescript were incubated with BrdU (1 h) during either early or late S after synchronization (see Methods), and cells were harvested 3 h later. ChIP (anti-Ac-H4) was performed on mononucleosomes and enrichment was determined for total plasmid sequences (which should be the same in both cell populations); early or late BrdU-labelled plasmids were isolated by immunoprecipitation of input (I) and bound (B) DNA with anti-BrdU. Eighty per cent of the transfected plasmid sequences are integrated in late-replicating regions. Enrichment (graph) was calculated relative to Ig-κ (set to 1.0) in each cell population.

cycle whereby endogenous chromatin structure is first disrupted and then reassembled in the wake of DNA synthesis[14,15]. The genomic replication timing profile of the genome itself seems to be set up independently of transcription[16,17] by means of *cis*-acting elements[1] and *trans*-acting factors[18–20] that affect the firing of local origins. As a result of this developmentally regulated organization and because the ability to set up transcriptional competence is dependent on S phase, early-replicating genes become assembled into an active structure, whereas late-replicating genes might be packaged into an inactive form during each passage through the cell cycle, thus providing a time-axis-based global mechanism for maintaining epigenetic states in dividing cells. □

Methods

Plasmids

PBluescript-based reporter constructs carrying the GFP or LacZ (β-galactosidase) coding sequence together with a nuclear localization signal were used to insert promoter regions for the mouse ribosomal protein gene S16 (208 base pairs; bp), the rat α-actin gene (800 bp), the mouse myogenin gene (1,100 bp) or the human EF1-α gene (1,207 bp) by PCR or restriction enzyme technology. S16-β globin was made by inserting the S16 promoter next to the human β-globin gene sequence[21], and O-S16–LacZ by inserting the *Eco*RI/*Bam*HI BPV origin-containing fragment derived from plasmid pKSO[22] into S16–LacZ at the *Not*1 site. The BPV-derived vector, B45-Neo, which lacks the genes E5, E6 and E7, was transfected into Rat-1 cells and stably maintained in episomal form at about 30 copies per cell[6].

Microinjections

Rat-1 cells (10^5) were seeded on 5.5-cm tissue culture plates containing gridded Cellocate disks (Eppendorf) 20 h before injection. Under these conditions each disk contains about 150–200 well-spaced cells, each of which can be identified from markings on the disk. Nuclear injection (0.4–1.5 ng μl^{-1} DNA) was performed as described[23]. Calibration of the system by using radioactive material showed that the average injection volume was 20 fl, containing 3–12 molecules of supercoiled plasmid DNA. For each plasmid we first performed trial injections at various concentrations to determine the amount of DNA that yielded suboptimal reporter gene activity (~50–60% LacZ- or GFP-positive cells). About 70% of the injected cells remained viable and divided normally in culture.

In experiments employing LacZ reporter genes, cells were fixed, usually 20 h after injection, and then stained with 5-bromo-4-chloro-3-indolyl-β-D-galactoside for 24 h before recording the positive reaction[24]. GFP-positive cells were detected by fluorescence microscopy 20 h after injection. However, identical results could be obtained by measuring GFP even 6 h after injection. It should be noted that only vital cells, those that had divided in the course of the experiment, were recorded. Injected DNA lacking an origin sequence remains unreplicated for at least 48 h as shown by restriction analysis with *Dpn*I and *Mbo*I before PCR. The BPV-origin-containing plasmid O-S16–LacZ, in contrast, underwent ~30% replication within 3 h of injection into B45-neo-transfected Rat-1 cells in either early or late S phase.

To programme injection schedules it was necessary to determine the cell cycle properties of these cells. We first determined the length of a full cell cycle by microscopic observation (on 100 cells) of the time from cell division to cell division. The lengths of G2 and S were then measured by incubating a non-synchronous population of cells with 5×10^{-5} M BrdU for 1 h, and then performing immunostaining[25] to detect incorporation into cells that divided at 2-h intervals after labelling. With this assay we found that uninjected cells have a cell cycle of 16 h (7 h G1, 7.5 h S, 1.5 h G2). A similar experiment on cells injected immediately after incubation with BrdU indicated that the injection procedure actually extends the cell cycle to 19 h (8 h G1, 9 h S, 2 h G2).

In some experiments cells were treated with Nocodozole (0.2 μM) and then synchronized at M/G1 by mitotic shake-off[26]. More than 80% of these cells then cycled normally and could be injected in either early or late S on separate plates. Monomeric and multimeric nucleosome fractions were prepared and ChIP analysis was performed with anti-Ac-H4 or anti-Ac-H3 (Upstate Biotechnology) to separate input from bound fractions as described[27]. Because ~1% of the nucleosomes are precipitated by this procedure, PCR of the bound fraction was always compared with a 1:100 dilution of the input DNA. To measure the acetylation state of chromosomal DNA, Rat-1 cells were stably transfected with pBluescript and grown as a pool of ~200 individual colonies. Cells were synchronized by mitotic shake-off (above) and then incubated with 10^{-5} M BrdU for 1 h in either early or late S phase. ChIP analysis was performed with anti-Ac-H4 and replicated sequences were specifically detected by immunoprecipitation of BrdU-containing DNA.

PCR analysis

PCR analysis of DNA was performed on LacZ (5′-ACGGCATGGTGCCAATGAAT-3′; 5′-GACCAGATGATCACACTCGG-3′, 127 nucleotides (nt), 36 cycles), endogenous β-actin (5′-CGCCATGGATGACGATATCG-3′; 5′-CGAAGCCGGCCTTGCACATG-3′, 68 nt, 21 cycles or 32 cycles for ChIP), β-globin (5′-GCTTCTGACACAACTGTGTTC-3′; 5′-CTGAAGTTCTCAGGATCCACG-3′, 450 nt, 36 cycles), Ig-κ (5′-AAACCTGCCTGAAGCCGAGC-3′; 5′-GATTGTGGGAGGAATGAGAA-3′, 76 nt, 32 cycles), pBluescript in Fig. 4 (primer I, 5′-GGGCTGCAGGAATTCGAT-3′, 90 nt; primer II, 5′-GGATCCCCCATCAAGCTTATCG-3′, 81 nt; T7, 5′-GTAATACGACTCACTATAGGG-3′, 40 cycles) and pBluescript in Fig. 5 (5′-CGCTCTAGAACTAGTGGATC-3′; 5′-TCGAGGTCGACGGTATC-3′, 72 nt, 38 cycles). RT–PCR[21] was performed on β-globin (as above, 352 nt, 36–39 cycles) and endogenous β-actin (5′-CGCCATGGATGACGATATCG-3′; 5′-CACGTAGGAGTCCTTCTGAC-3′, 166 nt, 23 cycles) after optimizing conditions. β-Globin and β-actin RNAs were detected with inter-exon primers. Quantification was performed by scanning autoradiograms or by PhosphorImager analysis and averaging the results for two or three different concentrations. To examine replicated O-S16-Lac Z plasmid specifically, DNA was treated before PCR with *Dpn*I (10 U μl^{-1}) for 4 h. Under these conditions, at least 85% of the bacterial DNA is digested (data not shown).

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Correspondence and requests for materials should be addressed to H.C. (e-mail: cedar@md2.huji.ac.il)

erratum

Reassessing the evidence for the earliest traces of life

Mark A. van Zuilen, Aivo Lepland & Gustaf Arrhenius

Nature **418**, 627–630 (2002).

On page 628, line 23, of this Letter, the isotope equilibrium fractionation temperature of 500 °C was incorrectly stated as 1,500 °C. □

corrigenda

Recovery of 16S ribosomal RNA gene fragments from ancient halite

Steven A. Fish, Thomas J. Shepherd, Terry J. McGenity & William D. Grant

Nature **417**, 432–436 (2002).

Figure 4b of this Letter included a sequence (Thailand-2 AJ319571), which we now realise is highly likely to be a chimaera between sequences Thailand-6 (AJ319575) and Thailand-7 (AJ319576), also shown in the phylogenetic tree. The chimaeric sequence is one out of twenty-three halite sequences shown in Fig. 4b, and did not occupy a pivotal position in the discussion; its inclusion in the phylogenetic tree therefore does not affect our general conclusions. The chimaeric sequence has now been removed from the databases. We are grateful to E. Willerslev and A. Cooper for bringing this to our attention. □

IRE1 couples endoplasmic reticulum load to secretory capacity by processing the *XBP-1* mRNA

Marcella Calfon, Huiqing Zeng, Fumihiko Urano, Jeffery H. Till, Stevan R. Hubbard, Heather P. Harding, Scott G. Clark & David Ron

Nature **415**, 92–96 (2002).

In this Letter, the commercial polyclonal antiserum used to detect endogenous XBP-1 was sc-7160 (Santa Cruz Biotechnology) and not sc-8015, as erroneously stated. The antibody sc-8015 is a mouse monoclonal and, in our hands, is not useful in detecting the endogenous protein by immunoblotting. □