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The DNA Content of Mouse Two-Cell Embryos Can Be Measured by Microfluorimetric Image Analysis Under Conditions of Cell Viability

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Video-enhanced fluorescence imaging was used to quantify the DNA content in live two-cell mouse embryos. DNA was stained with the vital fluorophore Hoechst 33342. Conditions of dye concentration and irradiation were such that two-cell embryos could be kept in the constant presence of the dye for about 24 h without a major effect on their further *in vitro* viability. Total nuclear fluorescence intensity of stained two-cell embryos was measured twice under these conditions, i.e., in G1 (1 h after cleavage) and in G2 (15–18 h after cleavage), by image analysis. After correcting for the fluctuations in excitation intensity and for the spatial nonhomogeneities of the optical system (lenses and sensor), the mean total nuclear fluorescence intensity was about twofold higher in G2 than in G1 ($\langle R \rangle = 1.99$ to 2.25), and this increase was abolished by the addition of aphidicolin, an inhibitor of replication. The fluorescence increase did not depend on the Hoechst concentration in the range of 10–40 ng/ml, i.e., in the range of embryo viability. The coefficient of variation of the total nuclear fluorescence intensity measured under these conditions was rather large (10 to 20%). Nevertheless, the mean value of fluorescence intensity in G1 of nuclei of a given pool represents an appropriate reference to measure the increase in fluorescence intensity between G1 and G2.

KEY WORDS: DNA quantification; fluorescence imaging; early embryos; video-enhanced microfluorimetry.

INTRODUCTION

When working on the dynamics of cellular events, it is necessary to know the position of the cell within its cycle as precisely as possible. Methods for direct quantification of DNA content by colorimetric or fluorescence staining have been developed (for review see Ref. 1). The analysis of the repartition of nuclear fluorescence within a cell population requires reliable quantitative methods. The most popular one is the flow cytometry, which proved very powerful for analyzing rapidly large populations and, in addition, sorting cells according to their DNA content (for review see Ref. 2). It is, however, not adapted for small populations.

The progress in video microscopy and image processing and analysis has pushed ahead the development of quantitative measurements based on fluorescence video microscopy [1,3,4]. The coefficient of variation for G1 intensities are generally higher than with flow cytometry (5 to 10 versus 1.5 to 6%), probably because of spatial inhomogeneities of the optical systems

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and the lack of linearity of the sensors [1,4,5]. Nevertheless, microfluorescence imaging requires limited manipulations, which can be implemented directly on cell cultures or tissues, without destroying the intercellular relationships. Furthermore, it allows simultaneous visualization of spatial and temporal patterns of other fluorescent cellular signals [5]. There have not been many studies dealing with quantitative fluorescence microscopy of the DNA content in living cells [6–9]. This may be due to the rather low penetrability of the dye, which requires high concentrations or long exposure times. These can lead, in the short- or long-term range, to deleterious effects [10]. Additional damage can be caused by irradiation during observation under light [11].

Recently we have shown that it is possible to perform direct microscopic observations of chromatin in live mouse embryos, without disturbing their cell cycle and further viability [12,13]. This methodology is based on the use of low concentrations of a DNA-specific and vital dye, Hoechst 33342, of very low irradiation intensities, and of digital video processing. The dynamics of chromatin changes can therefore be directly followed on oocytes and zygotes cultured *in vitro* in the continuous presence of the dye.

Under the same conditions, the total fluorescence intensity of stained nuclei is another measurable parameter at different phases of cell cycle. We have investigated if such measurements, obtained by video microscopy coupled to image digitization and analysis, could be reliably correlated to DNA content, under conditions of steady-state binding of nonsaturating dye concentrations. We performed this study on live two-cell mouse embryos, in which the two nuclei are still synchronous and spatially well separated, so that interferences between their respective fluorescence is minimal.

We demonstrate here that the total fluorescence intensity of stained nuclei measured in living cells by image analysis increases, on average, twofold between G1 and G2. This difference is abolished by the addition of aphidicolin (an inhibitor of DNA replication) in the culture medium. We have studied the effects of Hoechst concentration on variations in nuclear fluorescence intensity. These results are analyzed with reference to the further development of embryos to the blastocyst stage.

MATERIALS AND METHODS

Collection and Labeling of Embryos

Female C57/CBA mice 4–6 weeks old were superovulated with intraperitoneal injections of 5 IU of pregnant mare serum gonadotropin (PMSG; Folligon, Intervet), followed 48 h later with 5 IU of human chorionic gonadotropin (hCG; Chorulon, Intervet). Each female was caged with a C57/CBA male after hCG injection. A vaginal plug indicated successful mating. Superovulated eggs were collected from the ampulla at 22 h post hCG injection (hpHCG). Cumulus cells were removed by incubation in 1200 NF units/ml hyaluronidase (bovine testis type IV S, Sigma) in phosphate-buffered medium (PBI) containing 4 mg/ml bovine serum albumin (BSA) for 1–2 min at 37°C. Eggs were washed six to eight times in PBI-BSA.

Except when otherwise stated, eggs were then cultured in drops of Whitten's medium [14] containing 10 to 20 ng/ml of Hoechst 33342, maintained at 37°C under paraffin oil, in humidified air containing 5% CO_2. At 29–30 hpHCG, zygotes were examined by bright-field microscopy at half-hour intervals to assess their cleavage. Two-cell embryos formed within the previous half-hour were picked off and assembled in a synchronized pool. At 0.75–1 h post pickoff (hppo), two-cell embryos of a given pool were distributed one each in small droplets of the same medium containing Hoechst under oil, and the nuclear fluorescence intensity was measured for each blastomere. This allowed to measure each blastomere once and only once. This procedure was repeated at 15–18 hppo. Between the two measurements, embryos of each synchronized pool were reassembled for culture in the presence of the dye.

^{3}H-Thymidine Incorporation

DNA replication was measured by ^{3}H-thymidine incorporation. For that purpose, ^{3}H-thymidine (CEA; [*methyl*-^{3}H]thymidine, 49 Ci/mmol) was added at selected times after pickoff to the culture medium, either at 1 μCi/ml for 1-h pulse or at 0.1 μCi/ml for continuous incorporation. ^{3}H-Thymidine incorporation was stopped by extensive washes in PBS. Eggs were then placed on a slide and fixed in methanol at −20°C during 10–15 min. They were covered with a photographic emulsion (Amersham LM1) and kept in the dark for 3 weeks. The emulsion was then revealed and silver grains counted over the nuclei.

Egg Viability

Hoechst-stained embryos were washed after the last observation by six to eight passages in Whitten's medium to remove the dye. They were then cultured all together during several days in the same drop of Whitten's medium in the absence of Hoechst. In each exper-

iment, two control sets were kept. One was cultured starting with collection at the one-cell stage without Hoechst. The other was cultured from collection to 15–18 hppo in the continuous presence of Hoechst (10 to 20 ng/ml) but not irradiated. Egg viability was determined in each set as the percentage of cleavage to four-cells (ρ) as well as the percent of development to blastocysts (τ).

Fluorescence Microscopy and Image Registration

Stained eggs were observed by epifluorescence, with a Nikon Diaphot inverted microscope, using the commercial UV2A combination of filters and dichroic mirror. The microscope is equipped with an 100-W mercury lamp (OSRAM HBO 100), a thermostable box, and a system to allow flushing a mixture of 5% CO_2, 20% O_2, 75% N_2 over the surface of the petri dish. Additional neutral attenuators are set before the filter block so that a 2000-fold attenuated excitation beam is generally used. Each irradiation lasts less than 15 s.

Fluorescent images, generated by a $\times 20$ objective (NA = 0.75) and a X5 projective, are recorded on an intensified videocamera (Type 4336 Lhesa Electronique, Cergy Pontoise, France) with a manually adjustable gain and further digitalized on 8 bits (256 gray levels) by the SAPPHIRE system (Quantel Micro Consultants, Montigny le Bretonneux, France) as already described [12] (Fig. 1a).

Procedure for Quantitative Analysis

The dose–response of the whole setup was determined by measuring the intensity emitted by a standard fluorescent crystal (Zeiss GG17) at different attenuations of the excitation beam produced by a set of calibrated neutral attenuators. At each gain of the videocamera, the response is linear between 30 and 240 gray levels of the intensity scale.

Each experiment was performed without turning off the mercury lamp and with a strictly constant gain of the videocamera. Therefore, neutral attenuators were eventually used to keep all nuclear fluorescent intensity values of one experiment within the frame of this linear response. Variations in the intensity of the excitation beam, due to the relative instability of the mercury lamp, were periodically measured with the same standard fluorescent crystal. The nuclear intensities were then corrected for these fluctuations.

The spatial inhomogeneities due to the excitation light source and the response of the videocamera were removed by shading correction. For that purpose, a black and a white image are registered in each experiment and used as references. The shading correction was applied directly, through the SAPPHIRE system, on each digitalized image before quantitative analysis.

Quantitative intensity measurements were made using the image analysis program of the SAPPHIRE system. The nucleus outline is determined both by manual drawing and by thresholding the intensity (Fig. 1b).

Expression of Results

For intensity and surface measurements, mean values $\pm$ standard deviations are given. The coefficient of variation (CV) is defined as the standard deviation divided by the mean value. Differences in viability are analyzed by the chi-square test. Whenever possible (independent samples), mean values are compared by Student's t test.

RESULTS

Assessment of the Nuclear Stage

Two-cell embryos treated with a 1-h pulse of ^{3}H-thymidine (1 μCi/ml) at the pickoff, at 4 hppo, and at 15 hppo, respectively, showed different incorporation rates. When the pulse began at the pickoff, eggs showed no incorporation of ^{3}H-thymidine, except for a few of them. When eggs were treated at 4 hppo, they had a high incorporation rate. In contrast, no incorporation was observed after 15 hppo.

In another experiment, two-cell embryos were cultured in the continuous presence of 0.1 μCi/ml ^{3}H-thymidine from the pickoff until 1 and 12 hppo, respectively. Results were shown in Table I. In the second set, 77% of embryos generally ($n = 26$) showed an incorporation rate at least 20-fold higher than that of the first one. At the opposite, eggs treated with the same concentration of ^{3}H-thymidine from 12 to 15 hppo ($n = 20$) did not show any incorporation, except for one of them.

Taken together, these results show that eggs are in G1 or beginning of S phase at 1 hppo, while in G2 phase at 12 hppo. For those which are at the beginning of S at 1 hppo, the percentage of DNA already replicated does not exceed 5% the total replicated DNA.

Time Course of Hoechst Binding to DNA in Live Two-Cell Embryos

The time course of Hoechst binding to DNA in live two-cell embryos was measured in G2 phase, where the

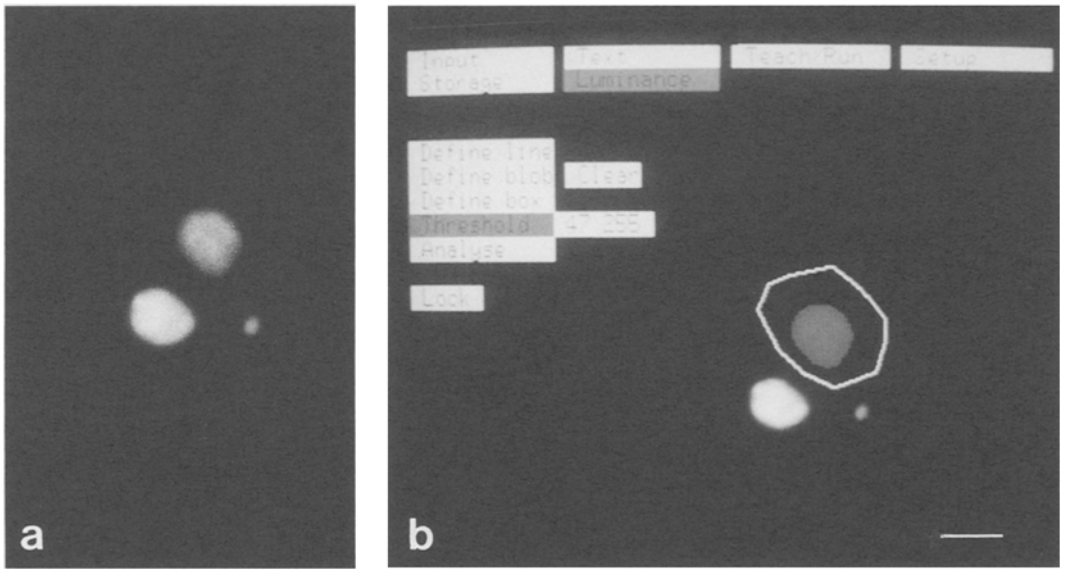

Fig. 1. Nuclear intensity measurement of live two-cell mouse embryo. Bar = 20 μm. (a) Digitalized image of nuclei at the G1 state as obtained by the video setup. (b) The total nuclear fluorescence intensity is measured in the circled area determined both by manual drawing and by thresholding the intensity.

Table I. Incorporation of ^{3}H-Thymidine in Two-Cell Mouse Embryos[a]

Beginning–end of ^{3}H-thymidine incorporation (hppo)	Concentration of ^{3}H-thymidine (μCi)	Number of cells observed	Level of ^{3}H-thymidine incorporation			
			−	±	+	++
0–1	0.1	22	17	5	0	0
0–12	0.1	26	0	0	6	20
12–15	0.1	20	19	1	0	0

[a] Background is estimated with unfertilized oocytes blocked at metaphase II, over a circular surface of 200 μm^2 (i.e., equivalent to that of a G1 nucleus). Background = 12 ± 3 grains (n = 12). (−) Estimated grain count equal to background; (±) estimated grain count 2 to 3 times over background; (+) estimated grain count 10 to 15 times over background; (++) estimated grain count at least 20 times over background.

DNA content is constant over a long period [15]. For that purpose, unstained embryos were distributed individually at 12 hppo in small medium droplets containing Hoechst (10 to 20 ng/ml), and fluorescent measurements were performed periodically on each blastomere.

The normalized time course of G2 nuclei labeling is shown in Fig. 2a. As seen on this figure, the nuclear fluorescence intensity increases up to a plateau. The use of an exponential fit suggests that the plateau is reached after 6 h labeling at 20 ng/ml. At 10 ng/ml, the plateau is nearly reached (up to 95%) after 8 h of staining. The fluorescence intensity increase is faster (Fig. 2a), and the maximal intensity higher (Fig. 2b), in the presence of 20 ng/ml than in the presence of 10 ng/ml. In addition, the G2 fluorescence intensity after 8 h of staining is the same as after 24 h of staining: the mean normalized values are, respectively, 1 ± 0.21 (n = 46) and 1.08 ± 0.23 (n = 46), the difference being not significant. Therefore the observed plateau represents the true maximal binding of Hoechst at that concentration and is not followed by a subsequent slow increase in fluorescence, which could have escaped a short-term observation.

Figure 2b shows two additional features. First, blastomeres with a lower fluorescence intensity remain less intense, even at the maximum, and vice versa. Second, two blastomeres of the same egg generally have nuclear fluorescence close to each other.

The time course of Hoechst labeling cannot be mea-

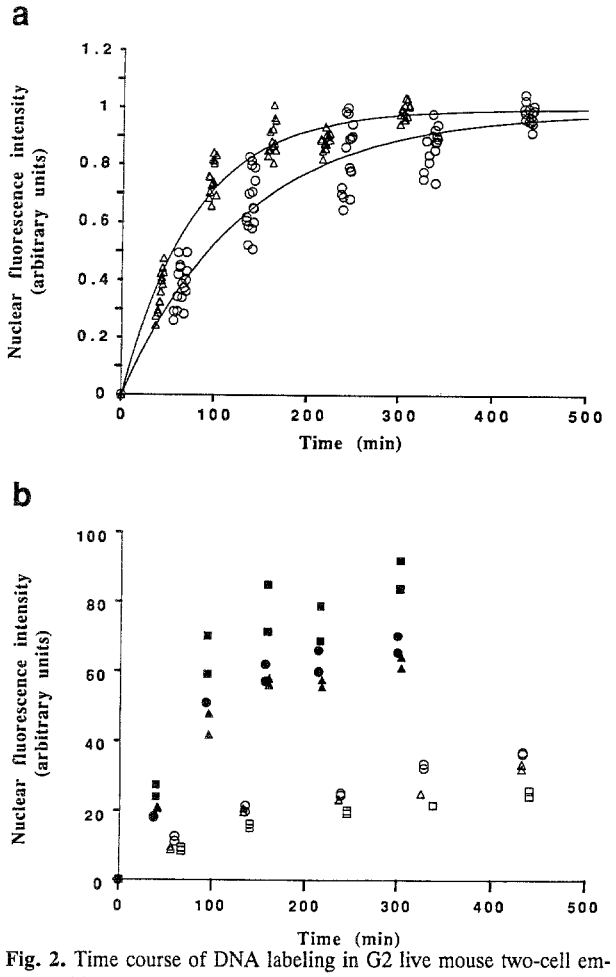

Fig. 2. Time course of DNA labeling in G2 live mouse two-cell embryos. (a) Data expressed in normalized values. The Hoechst concentration was 10 ng/ml (○) or 20 ng/ml (△). For each Hoechst concentration, 16 nuclei were monitored individually. The maximal nuclear fluorescence intensity (i_{max} = 1 in arbitrary units) was estimated through an exponential fit. (b) Data expressed in absolute values: 10 ng/ml (open symbols) and 20 ng/ml (filled symbols) Hoechst. Identical symbols refer to the two blastomeres of the same embryo.

sured on G1 nuclei because this stage is too short. We therefore exposed embryos to Hoechst in the zygote stage at least 8 h before the first fluorescent measurement (0.75–1 hppo) (see Materials and Methods) and assume that this is sufficient for each nucleus to achieve maximal DNA binding at that stage.

Egg Viability

We have first assessed the effects of both long culture in the presence of Hoechst (24 to 30 h) and repeated irradiations on embryos development, when they are cultured in pools. The results are shown in Table II. Our conditions of egg staining and observation (i.e., irradiation) decrease significantly the development rates, both to the four-cell (ρ) and to the blastocyst stage (τ) (Table II). Indeed, irradiated embryos (n = 114) stained with 10 ng/ml Hoechst have ρ and τ values, respectively, equal to 85 and to 67%, versus 97 and 88% for controls (i.e., neither labeled nor irradiated) (n = 120).

Two-cell embryos stained with 10 ng/ml Hoechst but not irradiated have a τ value equal to 70% (n = 145). This developmental rate is significantly lower ($P < < 0.01$) than that of controls but not as low as that of stained and irradiated embryos. Therefore, the length of culture in the presence of fluorophore seems to be the major factor leading to a decrease in the rate of development.

Embryos cultured individually have a poor development. Indeed only 45% of control unstained embryos, cultured individually from their pickoff, reach the four-cell stage and 20% the blastocyst stage ($n = 30$). Similarly, when Hoechst-stained and irradiated two-cell embryos are cultured individually from the pickoff, their developmental rate is very low: only 24% develop the four-cell and 4% to blastocyst stage ($n = 55$).

Cell Cycle Dependence of the Nuclear Fluorescence Intensity

From now on, the nuclear fluorescence intensity measured, respectively, at 0.75–1 h ppo (i.e., in G1 or beginning of S) and at 15–18 h ppo (i.e., in G2) was called i_1 and i_2, respectively. To allow good development (see above), embryos were cultured in synchronized pools between the two measurements of their nuclear

Table II. Viability of Two-Cell Embryos Cultured Under Various Conditions

Egg culture	Conditions	Number of embryos	Developed to 4 cells (%)	Developed to blastocyst (%)
In pools	Unstained, nonirradiated	120	96.7	88.3
	Stained 24 h, nonirradiated	145	88.3	70.3
	Stained 24 h, irradiated	114	84.5	66.7
Individually	Unstained, nonirradiated	30	45	20
	Stained 24 h, irradiated	55	24	4

fluorescence intensity. Therefore nuclei could not be individualized and each i_2 value could not be compared to its corresponding i_1 value for the same nucleus. We therefore had to compare i_2, either to the mean of i_1 ($\langle i_1 \rangle$) for all nuclei of the experiment or to the mean of i_1 for the nuclei of the synchronized pool to which it belongs ($\langle i_1 \rangle p$). The corresponding "apparent" ratios which characterize the fluorescence intensity in G2 of a given nucleus with respect to the fluorescence intensity in G1 are called, respectively, $R_p = i_2/\langle i_1 \rangle p$ and $R = i_2/\langle i_1 \rangle$.

When eggs are stained with 10 to 20 ng/ml Hoechst, i_1 values for all nuclei analyzed in a given experiment are distributed with a CV of 12–19% (Table III). The distribution of i_2 values for the same blastomeres is generally slightly higher (CV = 14–22%) (Table III). Above all, it is centered around a mean value ($\langle i_2 \rangle$) which is approximately twice the mean i_1 value ($\langle i_1 \rangle$). In other words, the mean value of R ($\langle R \rangle$) ranges between 1.99 and 2.25. Figure 3 shows the histograms of i_1 and i_2 values obtained from all experiments with 10 ng/ml Hoechst and with all $\langle i_1 \rangle$ normalized to unity ($n = 112$).

At the level of a synchronized pool, which can contain as low a number as four nuclei, the $\langle i_1 \rangle_p$ and $\langle i_2 \rangle_p$ values are again different, with $\langle R_p \rangle$ ranging between 1.78 and 2.66. Within each experiment, the $\langle R_p \rangle$ values themselves are distributed with a CV ranging between 7 and 13%. The wide distribution of the $\langle R_p \rangle$ values is not due to the noncorrespondence in i_1–i_2 couples, as shown by the following experiment. Two-cell embryos were cultured individually from their pickoff to blastocyst stage, taking into account only the few of them which developed to four-cell stage ($n = 12$), we found $\langle R_p \rangle$ values distributed between 1.78 and 2.74, with a CV of 13%. Note that in this case, $\langle i_1 \rangle_p$ represents the mean i_1 value between the two blastomeres of the same embryo. This result shows that working with individualized two-cell embryos does not improve the $\langle R_p \rangle$ value distribution.

To ascertain the relationship between nuclear fluorescence intensities and DNA contents, a set of Hoechst stained embryos was cultured in the presence of aphidicolin (10 μg/ml), an inhibitor of replication, added just after the pickoff. Nuclear fluorescence was measured twice as above. Under these conditions, the i_1 values of nuclei treated with aphidicolin were similar (main value and distribution) to those of nuclei from embryos not treated with aphidicolin and observed at the same time (Table IV). However, aphidicolin-treated embryos did not undergo any increase in their nuclear fluorescence intensity: in two separate experiments $\langle R \rangle$ values were, respectively, 1.03 ± 0.16 and 1.10 ± 0.22 (Table IV). In addition, aphidicolin added for 1 h after 15–18 h of culture in the presence of Hoechst did not modify the mean fluorescence level measured at that time. This excludes a direct effect of aphidicolin on Hoechst fluorescence.

As nuclei increase in volume between G1 and G2, we had to ascertain that this would not be the cause of the increase in the measured total nuclear fluorescence. Nuclei observed at 0.75–1 hppo appear generally round in the observation plane. The mean area $\langle s_1 \rangle$ of their

Table III. Cell Cycle Dependence of the Nuclear Fluorescence Intensity[a]

Hoechst (ng/ml)	n	CV_1 (%)	CV_2 (%)	$\langle R \rangle$	SD	n_p	$\langle R_p \rangle$	SD	Hoechst (ng/ml)	n	CV_1 (%)	CV_2 (%)	$\langle R \rangle$	SD	n_p	$\langle R_p \rangle$	SD
20	30	17.9	21.3	2.25	0.48	8	2.23	0.43	10	30	17.1	21.8	2.21	0.48	6	1.78	0.15
						4	2.09	0.39							4	1.98	0.36
						6	2.66	0.30							6	2.31	0.23
						12	2.07	0.44							4	2.38	0.16
20	22	19.2	21.8	1.99	0.43	4	1.92	0.25							10	2.37	0.30
						4	1.97	0.43	10	56	14.3	18.4	2.09	0.38	10	1.99	0.40
						4	1.92	0.41							16	2.06	0.38
						6	2.21	0.39							18	2.12	0.35
						4	1.84	0.30							6	2.34	0.49
20	38	17.3	15.1	2.19	0.33	6	2.24	0.19							6	2.00	0.33
						4	2.14	0.40	10	26	11.6	14.4	2.18	0.31	4	2.51	0.29
						6	2.57	0.42							4	2.21	0.27
						12	2.13	0.32							4	2.16	0.27
						6	2.21	0.42							14	2.09	0.36
						4	1.93	0.26									

[a] CV1 and CV2 are the coefficients of variation of the i_1 and i_2 distributions, respectively. n_p and n are the number of nuclei in each synchronized pool and in each experiment, respectively. $\langle R_p \rangle$ and $\langle R \rangle$ are defined in the text.

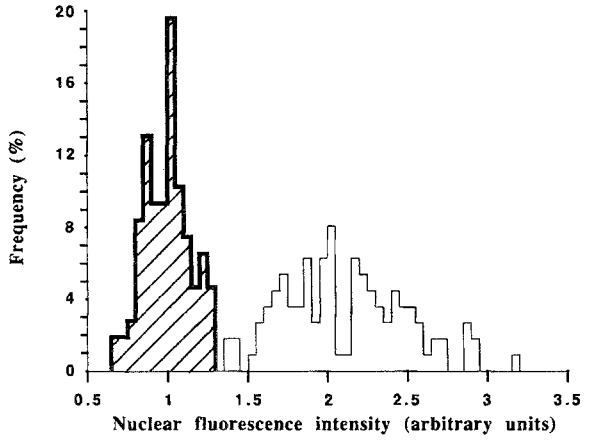

Fig. 3. Histograms of the nuclear fluorescence intensity distributions in G1 (hatched) and G2 for nuclei (n = 112) labeled with 10 ng/ml Hoechst. As i_1 and i_2 values were obtained from different experiments, $\langle i_1 \rangle$ values are normalized to unity.

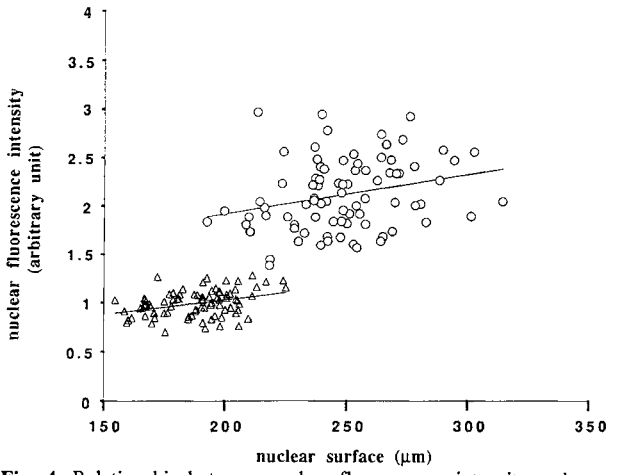

Fig. 4. Relationship between nuclear fluorescence intensity and nuclear optical section area for blastomeres observed both in the G1 (△) and in the G2 stage (○) and labeled with 10 ng/ml Hoechst.

Table IV. Effect of the Presence of Aphidicolin in the Culture Medium on the Total Nuclear Fluorescence of Two-Cell Embryos Measured at 15–18 hppo[a]

Hoechst (nM)	n	CV_1 (%)	CV_2 (%)	$\langle R \rangle$	SD	n_p	$\langle R_p \rangle$	SD
16	46	17.3	15.6	1.03	0.16	8	1.04	0.12
						14	0.92	0.13
						16	1.19	0.21
						8	0.95	0.16
16	26	17.1	20.1	1.10	0.22	4	1.26	0.12
						10	0.99	0.15
						12	1.06	0.20

[a]Abbreviations are as in Table III, footnote *a*.

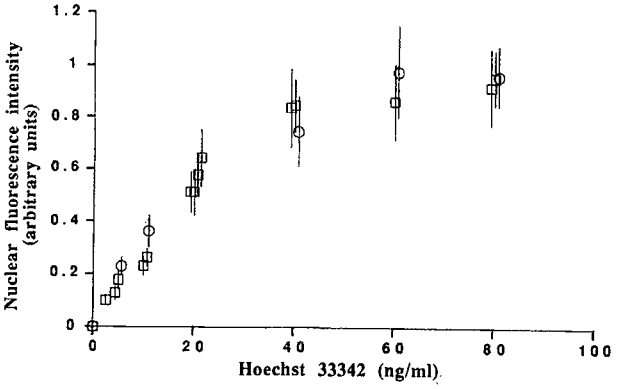

Fig. 5. Hoechst concentration dependence of the maximal fluorescence intensity for G2 nuclei stained during 24 h (□) or 8 h (○). Each experimental population (n = 18 to 30) is characterized by its mean nuclear fluorescence intensity ± SD (bar).

optical section is equal to 188 ± 16 μm² (n = 82). The same nuclei observed 15–18 hppo are more oval and larger. At that time, the mean area $\langle s_2 \rangle$ of their optical section is higher: $\langle s_2 \rangle$ = 247 ± 25 μm² (n = 82). In fact nuclear fluorescence intensity is slightly correlated with nuclear area in G1 but not in G2 (coefficients of correlation, respectively, 0.36 and 0.26). As shown in Fig. 4, the slope of the correlation line in G1 cannot account for the approximatively twofold increase in the nuclear fluorescence intensity between G1 and G2. In addition, aphidicolin-treated eggs exhibit the same increase in the nuclear volume between 0.75–1 and 15–18 hppo (n = 56; $\langle s_1 \rangle$ = 195 ± 22 μm²; $\langle s_2 \rangle$ = 243 ± 18 μm²) without concomitant variation of the nuclear fluorescence intensity.

Effect of the Hoechst Concentration

Measurements were performed on G2 nuclei, stained at least 24 h with several Hoechst concentrations. Results show that the nuclear fluorescence intensity increases with Hoechst concentration and reaches a plateau for approximately 80 ng/ml (Fig. 5). The concentration dependence of the maximal fluorescence intensity is similar in nuclei stained for only 8 h. Therefore saturation of chromatin with Hoechst seems to be achieved for concentrations of about 80 ng/ml. We must, however, point out that at concentrations higher than 60 ng/ml, failures in chromosome segregation at the first and sec-

ond cleavage begin to appear. These concentrations are therefore not suitable for embryonic development.

As we limited Hoechst concentrations to levels lower than saturation, we had to ascertain whether the Hoechst concentration would not be limiting the nuclear staining when DNA duplicates. We therefore determined $\langle i_2 \rangle$ values on eggs labeled continuously with various Hoechst concentrations. As seen in Fig. 6, the $<R>$ values remain approximately 2 in the range of 10–40 ng/ml and are lower at 80 ng/ml Hoechst.

DISCUSSION

The present study shows that (i) under conditions of nonsaturating Hoechst concentrations, nuclear fluorescence intensity of two-cell embryos reaches a steady-state level within 6–8 h, (ii) this level is reliably correlated with DNA content, and (iii) video-enhanced fluorescence imaging allows reliable quantitative measurements of this intensity, directly performed on the microscope stage, and with minimal effects of long-term cell viability. Therefore, the position of individual blastomeres within their cell cycle can be assessed for the first time in live embryos by rapid straighforward measurements, which, in addition, permit further development.

In the literature, quantitative studies are generally performed on cell cultures. In that situation, the position of a given cell within its cycle is not known. A preliminary statistical analysis of the fluorescent distribution among a rather large sample is necessary before being able to position a given cell with reference to the calibrated G1 and G2 intensity values. In the present experiment, the situation is opposite: the beginning of the cell cycle is monitored separately for each balstomere, which is analyzed twice (in G1 and G2) during its cycle. However, due to other constraints, each nucleus cannot be individualized between the two measurements, so that fluorescence variations with DNA content cannot be directly measured, and averaging i_1 and i_2 values is necessary for data analysis.

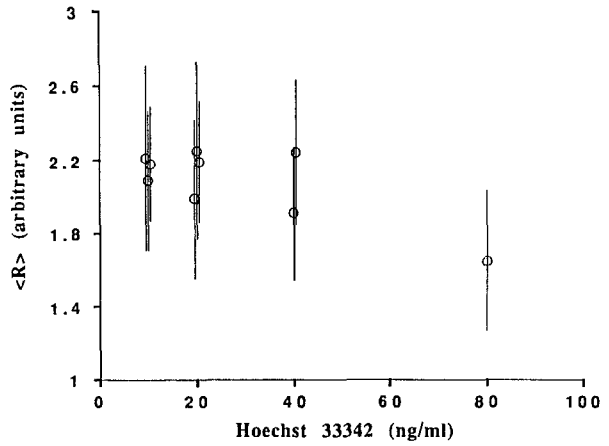

Fig. 6. Hoechst concentration dependence of the $\langle R \rangle$ value. Each experimental population (n = 18 to 56) is characterized by its $\langle R \rangle$ value (○) ± SD (bar).

Mouse embryos are apparently favorable objects for using Hoechst staining to determine DNA content under conditions of viability. Other cell lines may require much higher concentrations to lead to uniform uptake across the cell cycle and, therefore, to a normal G1, S, and G2 distribution [6]. In some cases, these concentrations may even be toxic [10]. Two-cell embryos have been chosen as a model because nuclei are synchronous and spatially separated, so that interferences between their respective light emissions are minimal. This situation is met neither in zygotes nor in embryos of stages later than four cells. Our conditions of embryo staining and irradiation have a negative effect upon egg viability. However, the decrease in development of irradiated embryos was highly consistent between replicates. Therefore, we are confident that our experimental conditions do not induce severe impairment in development.

Our results show that G1 nuclei of live mouse two-cell embryos have fluorescence intensities distributed with a CV ranging between 12 and 19%. Previous studies, dealing with other cell lines but the same fluorophore, reported intensity distributions of G1 nuclei less than those obtained here. CVs in the range of 5% have usually been obtained by flow cytometry [1,7], and slightly higher CVs (5 to 11%) with image analysis of fixed cells [1,4]. Several factors could be responsible for the rather wide fluorescence intensity distribution that we obtained. Some are linked to the method employed (video-enchanced fluorescence imaging with a Newvicon camera), as, for example, spatial nonhomogeneities of the optics and sensors, nonlinearity of the sensors, temporal fluctuations in the excitation light, and uncertainty in the estimation of nuclear outline. Others are linked to the cellular system under investigation and the requirements for its live state, such as, for example, the size of the blastomeres and physiological differences in permeability to Hoechst.

The nonlinearity of sensors has been put forward by many authors as one cause of wide distribution [1]. However, other authors have recently shown that the CV obtained on nuclear fluorescence by video-microfluorimetry are not significantly different between a CCD and a videocamera tube [4]. In the present study, measure-

ments were made under strictly constant gain of the videocamera, in the range of its linear response (see Materials and Methods). In addition, a shading correction was performed at the same gain to suppress spatial inhomogeneities of the optics and sensors. An uncertainty remains on the choice of the nuclear outline, whether carried out by thresholding the intensity level or by manual drawing. This one can be minimized by performing measurements by the same experimenter. The intensity of the excitation light fluctuates over the time of the experiment with a CV of ca. 7%, as revealed by repeated measurements with a fluorescent crystal. Nuclear fluorescence intensities are corrected to these fluctuations (see Materials and Methods), but in the absence of synchronous intensity monitoring, we cannot exclude higher-frequency fluctuations.

The thickness of both blastomere and nucleus (respective diameters, 50–60 and 15–18 μm) increases the internal filter effect of the excitation light, the diffusion, and the reabsorption of the emitted light. These effects depend also on the relative position of the nucleus with respect to the objective lens and could partially contribute to the slight correlation between nuclear size and fluorescence intensity observed in G1. The low numerical aperture (NA = 0.75) and low magnification (×20) of the objective used should allow collection of the maximum of light emitted by the nucleus. The cytoplasm itself has a weak fluorescence, about 10 times lower than that of the nucleus. Whatever its origin (free Hoechst or mitochondrial DNA), its contribution to the total fluorescence measured over the surface of the nucleus should be negligible, owing to the decrease in the optical transfer function when increasing the distance to the focal plane.

Another contribution to the high CV could be the difference in dye uptake between embryos. Indeed, the time course of Hoechst binding in G2 shows that nuclei with a lower (resp. higher) nuclear intensity are constantly less (resp. more) intense, while their $t_{1/2}$ values are not signficantly different from one another (Fig. 2b). Such differences in dye uptake have already been reported in previous studies. T and B lymphocytes could be separated on the basis of their fluorescence intensity in the presence of a given Hoechst concentration [16]. Lalande and Miller [17] have shown that allogeneic activation of lymphocytes results in an almost twofold increase in their main nuclear Hoechst fluorescence. All these studies suggest that Hoechst entry into mammalian cells could be an unmediated diffusion [18], while its efflux could be mediated by rapid efflux pumps [19,20]. Crissman *et al.* [21] showed that the combined use of a membrane potential modifying fluorochrome, DiO–C5-3, improved both the main Hoechst uptake and the CV on CHO cells. Therefore, the wide distribution of nuclear fluorescence intensity could result from different efficiencies of similar Hoechst transport mechanisms between embryos. In that context, it is interesting to note that intensity values of two blastomeres of the same embryo are generally very close, either in G1 or in G2. When the mean nuclear fluorescence of the two sister blastomeres is taken as a reference for each embryo, the nuclear intensities are distributed with a CV of 5–8% in G1 and 9–11% in G2, i.e., in the range of coefficients of variation reported by other authors. Therefore the difference between these CV values and those shown in Table III gives an estimate of the importance of the random factors (lamp, estimation of surface, depth of nucleus, difference in dye uptake between embryos) to the intensity distribution.

The eventual difference in Hoechst uptake is likely to have more influence on intensity distribution under nonsaturating conditions, which are those used in the present study. Indeed many authors [6,7,16] report a higher CV when using nonsaturating Hoechst concentrations. However, in the case of two-cell embryos, the concentration cannot be increased to more than 60 ng/ml without risk of some deleterious effects on cell viability ($\rho = 40\%$ at 80 ng/ml, vs 85% at 10 ng/ml Hoechst), especially through defects in chromatin segregation at mitosis. Therefore the present study was restricted to a narrow concentration range. It was particularly important to show experimentally that the Hoechst concentration range is not limiting, i.e., doubling the DNA content leads to a doubling of the fluorescence intensity. The demonstration of proportionality between fluorescence intensity and DNA content in all Hoechst concentrations studied is essential, all the more because the interaction of Hoechst with naked DNA *in vitro* is a complex mechanism [22,23]. In the case of nuclei, the presence of DNA binding proteins and the condensation state of chromatin should also be taken into account, and it is beyond the scope of the present work to study the Hoechst–DNA equilibrium on living cells. In the present study, saturation with Hoechst seemed to be achieved at about 80 ng/ml.

The fact that the fluorescence intensity ratios between G1 and G2, whether calculated with reference to the mean i_1 of the overall experimental population (R) or the mean i_1 of the synchronized pool (R_p), are distributed with similar CV indicates that it is possible to distinguish between G1 and G2 nuclei even when dealing with a small population of blastomeres, which is generally the case in the field of developmental biology. This is of great practical importance if one wishes to

discriminate the position in the cycle of individual asynchronous blastomeres issued from the same embryo at a later stage and keep them alive for further experiments.

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