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Fluorimetric DNA Assay for Cell Growth Estimation

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A growth assay using the fluorescent dye Hoechst 33258 has been developed which allows sensitive and rapid analysis of the DNA content of a variety of cell types including keratinocytes and mucus-secreting cells, and which requires a minimum of liquid handling. The assay can detect as few as 500 diploid human cells, and is compatible with the simultaneous detection of [^{3}H]thymidine incorporation in the same cultures.

Many assays exist for the determination of DNA in tissues or cultured cells, several of which use Hoechst dyes such as H33258[2] (bisbenzamide: [2-(4-hydroxyphenyl)-5-[4-methyl-1-piperazinyl]-2,5′-bi-1H-benzimidazole) or DAPI (4′,6′-diamidino-2-phenylindole) because of their superior sensitivity and specificity for DNA (1–6). Such assays are useful for the measurement of the proliferation of cells, since DNA content reflects cell number more accurately than other parameters such as dye take-up (7) or alkaline phosphatase estimation (8), which may not be universal in their experimental application [discussed in (9)]. We are not keen to estimate cell number by a method which can falsely attribute to a parameter significance where it is not due. For instance, when epidermal keratinocytes stratify, their protein content per cell increases greatly, but cell number need not change at all. Thus a protein assay could mislead one to think that cell number had risen. In addition, DNA assays vary by several orders of magnitude in their sensitivity (i.e., uv spectroscopy versus fluorescence), and fluorescent assays are not necessarily specific for DNA (i.e., propidium iodide or ethidium bromide versus Hoechst dyes), and so the choice of fluorochrome is important.

If so many assays exist for DNA, why produce another? We study several cell types, including fibroblasts, breast epithelial, and gastrointestinal tumor cell lines and keratinocytes. The latter two groups of cells respectively contain mucus or fibrous proteins which render them harder to disperse into solution for DNA estimation. We wanted a semiautomatic microassay which would accommodate all cells tested with a minimum of liquid handling. The assays referred to above had each some shortcomings for our requirements, and we believe our method will have general applicability since the number of steps involved is minimal, while the reliability of the data is high.

MATERIALS AND METHODS

Calf thymus DNA sodium salt, Hoechst 33258 (bisbenzimide), Tris (tris(hydroxymethyl)aminomethane), and Triton X-100 (octyl-phenoxy polyethoxyethanol) were bought from Sigma (Poole, UK). Acridine orange was purchased from Molecular Probes (OR). Bovine pancreatic DNase 1 (grade 1) came from Boehringer Mannheim (Lewes, UK). Analytical grade sodium hydroxide and potassium dihydrogen orthophosphate were bought from Fisons (Loughborough, UK). Other chemicals were of analytical grade from BDH (Poole, UK). Clear 96-well plates for tissue culture were obtained from Becton–Dickinson (Oxford, UK). Black or white flat bottom 96-well plates for fluorescence analysis were brought from Dynatech (Billingshurst, UK). Investigators should be aware that DNA-intercalating drugs such as H33258 and AO are mutagenic, and the wearing of gloves should be strictly observed and aerosol formation avoided.

During the initial development of the assay fluorescence measurements were performed on either a Per-

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[2] Abbreviations used: AO, acridine orange; BSA, bovine serum albumin; Chaps, [(3-cholamidopropyl)dimethylammonio]propanesulfonate; DNA, deoxyribonucleic acid; EB, ethidium bromide; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; GuITC, guanidinium isothiocyanate; H33258, Hoechst dye [2′-(4-hydroxyphenyl)-5-[4-methyl-1-piperazinyl]-2,5′-bi-IH-benzimidazole; PBS-A, phosphate-buffered saline, without calcium or magnesium; SDS, sodium dodecyl sulfate; SSC, saline sodium citrate buffer; TX-100, Triton X-100.

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kin–Elmer LS5 (monochromator model with cuvettes) or an LS2 (filter model with flow cell). For microassays we used Fluoroskan II (Labsystems; 96-well plates only) and Cytofluor 2300 (Millipore; 96,48,24 or 6-well plates) plate readers. The Fluoroskan was keyboard driven and data were down-loaded to an Apple Macintosh SE computer for statistical and graphical analyses. We now use the analytical program "DeltaSoft" (Biometallics, Princeton, NJ), through which the plate reader can be controlled and the data manipulated. The Cytofluor was PC-driven, with spreadsheet analytical programs. It could only perform assays using clear plates.

SSC was made as a 20× stock and diluted before use to yield 0.154 M NaCl, 0.015 M Na_3 citrate, pH 7.0. SDS and Hoechst 33258 were made respectively as 10% (w/v) and 800 μg/ml stock solutions in "Milli-Q" distilled water. Hoechst 33258 was stored at −20°C. Working solutions were kept in foil-covered tubes to protect from light.

Cell Culture

Cells used in these assays included those derived from human pancreatic tumours (Panc-1, Aspc-1, Bxpc-3), human breast carcinoma cells (MCF-7), and normal human epidermal keratinocytes, prepared and cultured by the method of Otto *et al.* (10). Pancreatic cells were cultured in RPMI 1640 with 2% sodium bicarbonate and 10% fetal calf serum (FCS; GIBCO–BRL, Paisley, Scotland), while keratinocytes and MCF-7 cells were grown in DMEM with 10% FCS, which for the latter cell line was "stripped" by the method of Damassa *et al.* (11), with added pyruvate (1 mM final concentration), kanamycin (100 μg/ml; Winthrop, Guildford, Surrey, UK) and Fungizone (1.25 μg/ml; Squibb, Liverpool, UK). Worthington trypsin for routine subculture was bought from Lorne Diagnostics (Reading, UK). All cells were grown at 37°C in a humid incubator in an atmosphere of 5% (pancreatic and breast cells) or 10% (keratinocytes) CO_2. Tissue culture plastic was purchased from Becton–Dickinson.

Effects of Solubilization Procedure

The following solvents have been tested: SDS, NP-40, Chaps, GuITC, Tx-100, NaOH, Urea, EDTA. For each solvent, calf thymus DNA (ctDNA) was diluted from a stock DNA solution (10 mg/ml) made in 10 mM Tris (pH 7.4)/1 mM EDTA (pH 8.0) and stored frozen until needed. DNA concentrations were in the range 0–50 μg/ml. Concentrations of solvent were in the ranges 0–2% (w/v or v/v as appropriate) for SDS, Chaps, NP-40, and Tx-100 and 0–1 M for NaOH, GuITC, and EDTA, made up in SSC, PBS-A, or 10 mM Tris, pH 8.0 (4). Urea was added from a concentrated stock (16 M) or as solid. Standard curves were tested with fluors dissolved in the same solvent, or in distilled water, in the concentration range 0–10 μg/ml. "Standard curves" for cell lines were run in parallel using freshly trypsinized cells, diluted to give a range of cell suspensions from 500–500,000 cells/ml. The effects of temperature (ambient, 37 or 60°C) and time of incubation (up to 24 h) on dissolution were monitored by microscopy.

Growth Assay

Cells were grown in flasks until approaching confluence, trypsinized, and for initial experiments cell suspensions were pelletted (100*g*, 5 min) in plastic tubes prior to dissolution. Fluorescent measurements were made in 3-ml quartz cuvettes on dissolved cell suspensions (2 ml) after the addition of 1 ml of Hoechst dye (1 or 4 μg/ml). For semiautomatic plate reader experiments, cells were seeded at varying densities into 24-well (Cytofluor) or 96-well (Cytofluor or Fluoroskan II) plates in growth medium (1 ml or 100 μl/well for 24 or 96 wells, respectively), with certain experimental procedures as given below. At the end of the experimental period, medium was aspirated and the wells were washed twice with PBS-A. After removing this solution plates could be frozen pending analysis. Cells were exposed to test solvents for varying times and temperatures, after which an equal volume of SSC or other test buffer containing fluorochrome was added and the plate scanned by the machine at the respective excitation and emission wavelengths of 355 and 460 nm for Hoechst 33258 and 485 and 538 nm for acridine orange (wavelengths for DNA). All analyses using plate readers were performed *in situ,* unless liquid transfer (100 μl/well) to white or black plates was performed. Direct growth assays were attempted on γ-irradiation-sterilized (2.5 Mrad) samples of these non-tissue culture treated plates, but cell adhesion was not consistent, nor was it possible to assess the cells by microscopy, and such assays were not pursued. Manipulations were kept to a minimum.

Plate Readers

Scanning took around 30 s per plate using either machine. The advantage of the Cytofluor in its flexibility of analyzing many plate formats was reduced by its poor sensitivity when using the Hoechst dye (a new model has improved uv sensitivity). At visible wavelengths its performance was good, with a manufacturer's quoted sensitivity for fluorescein of 0.1 ng/ml. The Fluoroskan's uv sensitivity (quoted at 0.02 ng/ml 4-methyl umbelliferone, a fluor with similar wavelength maxima to Hoechst 33258) was primarily due to a xenon lamp. Both plate readers had a selection of excitation and emission filters to accommodate common fluors including fluorescein, rhodamine, texas red, or 4-methyl umbelliferone.

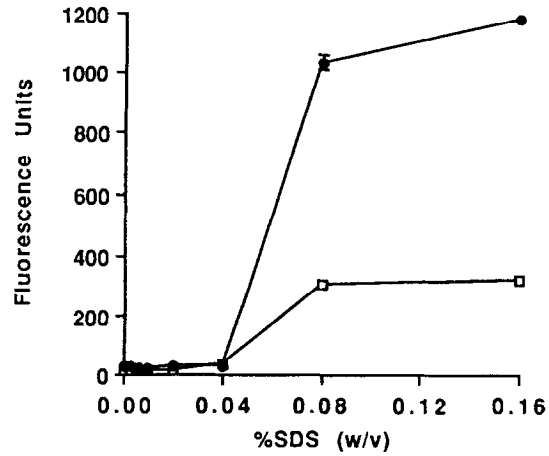

FIG. 1. Measurement of nonspecific fluorescence with increasing concentrations of SDS in the presence of 1 (open squares) and 4 (solid circles) μg/ml Hoechst 33258, respectively.

Reproducibility

The data (means ± SD unless too small to be depicted) presented are representatives of several experiments, in which replicates were never less than four, while for 96-well plates these were routinely eight. Regressing fluorescence against DNA concentration routinely gives R^2 values over 0.99.

RESULTS

Solvents

The use of all solvents caused some background fluorescence, especially at high concentrations. SDS above 0.05% was highly fluorescent, and this was increased with Hoechst 33258 at 1 or 4 μg/ml, but reagent blanks below 0.04% SDS gave a low background (Fig. 1). Cross-talk between wells was not a problem for any type of microtitre plate, as illustrated for the Fluoroskan II in Table 1. Wells containing SSC alone surrounded by empty wells did not differ in their endogenous fluorescence from wells with SSC surrounded by any concentration of Hoechst dye tested (0–4 μg/ml) (rows 1 and 2). The reagent blanks for the assay comprised SSC/SDS with added Hoechst dye in the absence of DNA. Table 1 also shows that these blank values depended on Hoechst concentration, but they were small compared to the values obtained in the presence of DNA (Fig. 2A). The appropriate blank values were subtracted from all subsequent readings for the calculation of DNA.

NP-40, Tx-100, and NaOH permitted the linear relationship between fluorescence and standard DNA concentrations to be seen, but the slopes of the regression lines were low. EDTA, pH 12.3 (2), was less efficient than SDS at cell dissolution, and gave a less sensitive standard curve of DNA fluorescence. Of the buffers used, SSC gave a lower background fluorescence than Tris and higher sensitivity than PBS-A. The highest sensitivity to DNA was found in 0.02% or less SDS (final concentration after addition of fluor), while the combination SSC/0.02% SDS was optimal and was chosen for microassay development. This system dissolved all moderate cell numbers (up to 200,000/ml of solvent) at RT. Higher cell numbers, or epidermal keratinocytes, required either heat treatment (37 or 60°C) or prolonged exposure to achieve dissolution (see below), and then samples were best diluted prior to reading since the linearity of the fluorescence did not hold without raising the fluor concentration to 4 μg/ml. High concentrations of SDS could be added to lyse high numbers of cells, but the final concentration, after Hoechst addition, should be below 0.04% when reading in the fluorimeter. It was important to avoid bubble formation when dispensing liquids for fluorimetry.

DNA Assay

Sensitivity to Hoechst dye was dependent on the machine used. Greatest sensitivity was seen using a Perkin–Elmer LS5 monochromator fluorimeter (1 ng/ml DNA), while high sensitivity was still seen in the Fluoro-

TABLE 1

Effect of Microtitre Plate on between-Well Cross-Talk and Blank Fluorescences

	Black	Clear	White
1. SSC/empty	0.175 ± 0.078	4.566 ± 0.446	1.827 ± 0.180
2. SSC/Hoechst	0.258 ± 0.170 NS	4.565 ± 0.382 NS	1.785 ± 0.299 NS
3. Hoechst 1 μg/ml	2.660 ± 0.293	6.090 ± 0.568	15.290 ± 1.759
4. Hoechst 2 μg/ml	3.856 ± 0.258	6.937 ± 0.311	18.130 ± 0.581
5. Hoechst 4 μg/ml	5.632 ± 0.299	8.291 ± 0.476	26.830 ± 0.750

Note. Row 1 contained SSC in wells surrounded by empty wells. Row 2 contained SSC in wells surrounded by wells containing Hoechst 33258. No differences were found between wells containing SSC surrounded by any concentration of Hoechst 33258 and data have been pooled. Rows 3–5 contained reagent blanks for the DNA assay with differing Hoechst 33258 concentrations. All wells contained 200 μl. All measurements were performed at λex/λem 355/460nm, and data are means ± SD ($n > 6$). NS, not significantly different ($P > 0.05$) from row 1 data by t test.

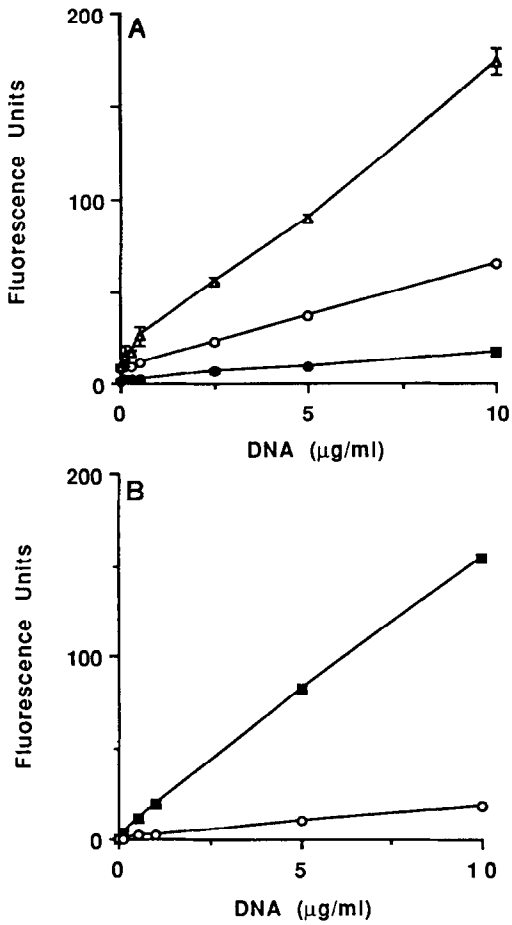

FIG. 2. (a) Comparison of DNA standard curves assayed with a Fluoroskan II plate reader in three 96-well plastic plates: white (open triangles), clear cell culture-treated (open circles), and black (solid circles). (b) DNA standards assayed using a Cytofluor 2300 plate reader (clear plates only) with Hoechst 33258 (open circles) and acridine orange (solid squares).

skan II, but this depended on the plastic plate used—white plates (5 ng/ml) were consistently superior to clear or black plates (Fig. 2a; but compare Fig. 5a for LS5 standards). The Cytofluor was less sensitive for Hoechst 33258 (300 ng/ml DNA could be estimated) but very good at the visible AO wavelength (Fig. 2b).

Binding of Hoechst 33258 to DNA was fast and stable, provided samples were protected from heat to prevent evaporation, or strong light to avoid bleaching. Higher sensitivity to DNA was consistently found with Hoechst 33258 over acridine orange with fluorimeters having xenon lamps, whether in DNA standard curves or in cell solutions. However, it should be recalled that no RNA assay is possible using Hoechst 33258 alone.

Binding of H33258 to DNA was DNase sensitive when assayed in Tris buffer, and DNase resistant when assayed in SSC/SDS (Fig. 3), indicating an advantage of this buffer system, particularly for keratinocytes which have high levels of nucleases when differentiated. The effect of large amounts of BSA (0.6 mg/ml) was slight in reducing the sensitivity of the assay.

Cell Assays

Pancreatic (Bxpc-3, Panc-1, and Aspc-1) and breast carcinoma (MCF-7) cell lines all gave linear responses to fluorescence with increasing cell number (Figs. 4a and 4b), while differential growth of MCF-7 cells grown on 96-well plates was demonstrable using low or high serum supplemented with estradiol (Fig. 4c).

Keratinocytes

Epidermal keratinocytes are inherently less soluble than simple epithelia due to their keratin content and mode of differentiation into squames with a cornified envelope. We tested several harsher solubilization regimes on these cells to enable the Hoechst assay to be performed. Pipetting up and down of keratinocytes assisted in their dispersal. GuITC (1 M) and 0.2–1% Chaps failed to dissolve keratinocytes separately or together. Urea alone (4 M) was not successful, but partial dissolution was achieved with 4 M urea with 0.5% Chaps added, while 8 M urea and above achieved complete dissolution immediately. SDS at 0.02% did not dissolve these cells even with heating to 60°C for 2 h, nor with 1% NP-40 added. However, 0.05% SDS dissolved most cells while leaving cornified cells intact. Standard DNA curves were linear with up to 16 M urea, but with less sensitivity than without (Fig. 5a). Addition of urea (16 M) to SSC/0.02% SDS was compatible with the Hoechst assay (Fig. 5b).

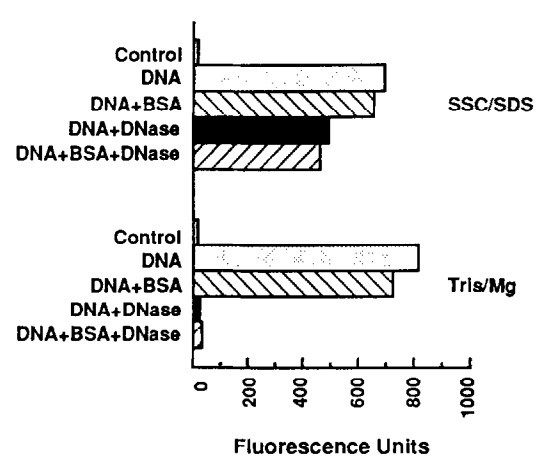

FIG. 3. Assay sensitivity to DNase. DNA (40 μg/ml) was dissolved in 0.02% SDS/SSC or 10 mM Tris, pH 7.4, containing 5 mM $MgCl_2$ in the presence or absence of BSA (60 μg/100 μl) and exposed to DNase (10 μg = 4.2 U/100 μl) at 37°C for 120 min. Readings were made in white plates.

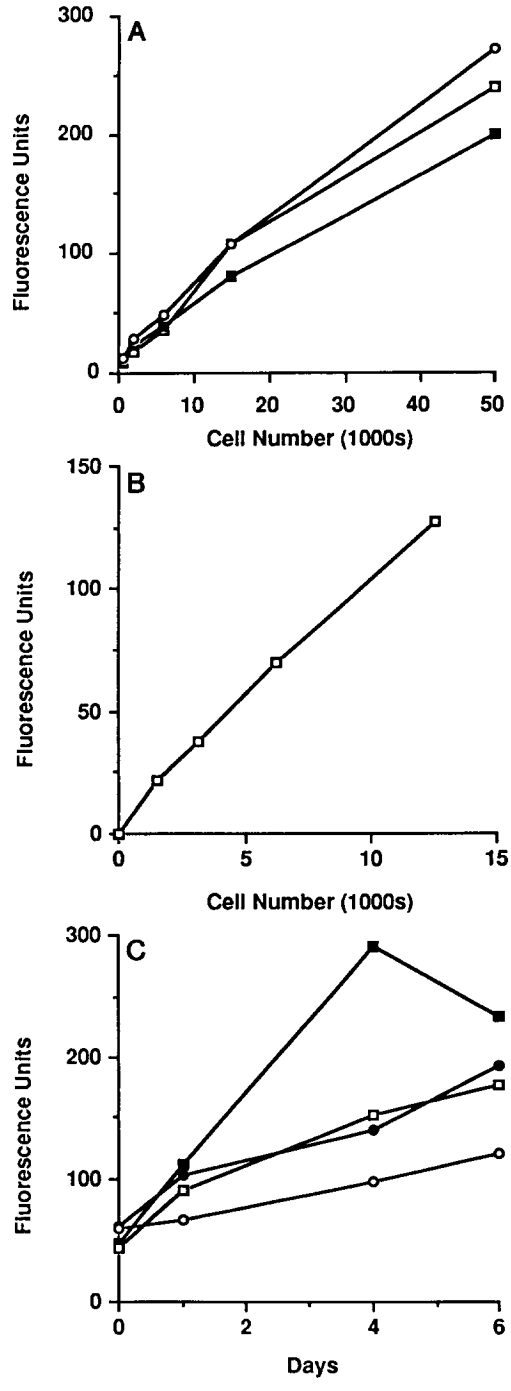

FIG. 4. Fluorescence dependence on cell number for: (a) pancreatic lines Bxpc-3 (open circles), Panc-1 (open squares) and Aspc-1 (solid squares). (b) MCF-7 breast carcinoma cells. (c) Growth of MCF-7 cells in 1% (open symbols) or 10% (solid symbols) stripped FCS in the absence (circles) or presence (squares) of 10 nM estradiol. All measurements made in white 96-well plates.

^{3}HThymidine Incorporation

A combined assay for the incorporation of tritiated thymidine (^{3}H-TdR) and DNA may be carried out with the following alterations to the method (data not shown). Cells are exposed to ^{3}H-TdR as a terminal 1- to 4-h pulse at 1 μCi/ml. Nonincorporated label is then removed by three PBS washes and the cells are assayed for DNA as above. Aliquots from each well can then be scintillation counted, or quantitatively transferred to filters using a cell harvester and assayed without scintillant (i.e., Berthold 2000 system). However, such estimations do not allow for any intracellular thymidine that has not been incorporated, and so data from cells for which evidence of a high intracellular pool or thymidine store is known (12) should be treated cautiously.

Simplified Assay Procedure

A brief protocol for the optimized DNA assay in a plate reader is given below:

1. Plate cells and treat as appropriate for *n* days to a maximum of 10^5 per well.
2. Aspirate medium from each well by gentle suction, or invert onto absorbant paper towels.
3. Gently rinse out each well twice with 100 μl PBS-A and drain. Plates may be stored frozen for later analysis at this stage.
4. To each well add 100 μl of 0.02% SDS (diluted in 1× SSC—include 8 M urea for keratinocytes).
5. Incubate plate at 37°C for 1 h with occasional swirling.
6. Add 100 μl of 1.0 μg/ml of Hoechst 33258 in 1× SSC.
7. Transfer 100-μl aliquots to a white 96-well plate.
8. Read fluorescence of each well at excitation λ 355 nm, emission λ 460 nm, comparing with DNA solutions or cell "standards."

DISCUSSION

Many assays exist for the estimation of DNA, and their sensitivities differ over several orders of magnitude. Ultraviolet spectroscopy at 260 nm can estimate above 500 ng/ml DNA, but suffers from interference due to protein or RNA. The diphenylamine assay of Burton (13) (10 μg/ml DNA) is not sensitive enough for microanalysis. Fluorimetry offers the highest sensitivity and fluors exist with complete DNA specificity, thus eliminating difficulties over RNA content (1), although this may be estimated fluorimetrically with acridine orange should one wish. The fluorescent DNA assay of Johnson-Wint and Hollis (14) using diaminobenzoic acid can measure down to 1 μg/ml. However, the assays with the greatest sensitivity to date have been those based on the Hoechst dyes and DAPI, with a lower detection of DNA of around 10 ng/ml (1–6).

The critical phase to any DNA assay is the dispersal of the cells to yield a solution. Our previous assay (10) required growth of cells in a 24-well plate together with homogenization of each well's contents using a Poly-

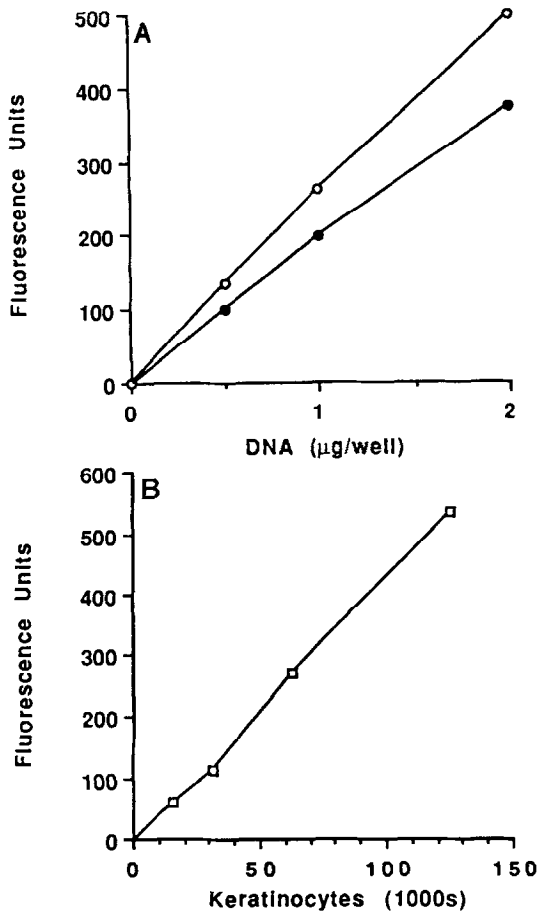

FIG. 5. (a) DNA standards in the absence (open circles) or presence (solid circles) of 16 M urea. (b) Keratinocytes assayed in the presence of 16 M urea. Measurements made using Perkin-Elmer LS5 fluorimeter.

tron, with proteinase and RNase treatment of the homogenate prior to fluor (ethidium bromide) addition. Although the assay could also discriminate ±10 ng/ml DNA it was laborious and noisy and not well suited to microanalysis or automation.

Semimicroassays can combine high sensitivity with small volumes of medium per well, and so can achieve an economy of peptides or other costly factors used as growth promoting agents, as well as rapid throughput. The 96-well format is convenient and rapid with automatic data handling via microplate readers. On this scale it is impracticable to measure cell number by trypsinization and counting, so the best alternative is cell DNA content, as this is the single constituent most likely to alter in proportion to cell number when culture growth is challenged. Assays purporting to measure culture growth by assaying for neutral red uptake by cell lysosomes (7), enzyme activities such as alkaline phosphatase (8), intracellular protein staining with crystal violet (15) cannot fulfill this paramount criterion, and results from such assays should be treated cautiously. Critiques of the use of [^{3}H]thymidine to measure cell growth (16–18) are also pertinent. However, our assay is compatible with the simultaneous use of radioactive techniques.

The choice of compounds that show specific enhanced fluorescence in the presence of DNA is essentially between DAPI and Hoechst dyes such as 33258. The Hoechst dye shows remarkably little influence of RNA on fluorescence and a linearity of fluorescence with a wide range of DNA concentrations (1). It also has some advantages over DAPI in terms of sensitivity and the need for an internal standard (19).

Labarca and Paigen (1) described the influence of histones on the fluorescence enhancement of Hoechst 33258 at different histone concentrations, and concluded that 2 M salt was optimal for DNA analysis. There was 60% reduction in fluorescence of tissue homogenates in the absence of salt but the absolute fluorescence enhancement was still considerable. We have found that there was little advantage in our system of opting for high salt concentrations, with a strong and linear signal obtainable from the salt concentration provided by SSC, in which the citrate will dissociate nuclear proteins from DNA while inhibiting DNase activity. Like previous authors (1) we found the fluorescence enhancement to be completely sensitive to DNase activity in Tris buffer, but that the SSC was suitably inhibitory to allow accurate analyses.

An assay has been reported for the simultaneous fluorimetric measurement of DNA using Hoechst 33258 and GAG content of cartilage tissue (4). This assay is highly reproducible, but is inevitably more lengthy than that reported here due to the starting tissue needing several processing steps (i.e., DNA extraction) prior to quantitation.

The assay recently reported by Rago *et al.* (5) is that most similar to the one reported here. We have found their method to be less reliable in two key areas. By relying on freeze–thawing of samples in distilled water, a highly particulate dispersal of cellular material ensues, which for keratinocytes and mucus-secreting cells results in a reduced sensitivity to the Hoechst dye. We have seen a threefold rise in sensitivity when using white rather than clear plates for assay of low cell numbers (compare the slopes of the DNA standards in Fig. 2A), and we estimate that the sensitivity of the Rago *et al.* assay would also benefit from this.

One further point regards the suitability of the different types of 96-well plates. Clear plates suffer from a low sensitivity for Hoechst dyes, and between-well interference cannot be ruled out, although our data suggest that it is already low. It is inappropriate to grow cells in white or black plates since no microscopy is possible, nor are they tissue-culture treated. We await the development of clear plates with fluorescence attributes that

are improved over those currently available so that these sensitive DNA assays can be performed entirely *in situ*.

Since these experiments were initiated, we have used the assay to measure the growth of a number of other cell lines, including normal human skin fibroblasts and dermal papilla cells (R. Dover, ICRF, personal communication), an additional range of human breast epithelial tumor cell lines (BT-20, ZR-75, T47D), human colorectal carcinoma cells (COLO-357, Col 29 [a subline of HCA-7 (20)], HRA-19 (21)), and a human gastric carcinoma cell line (HGT-101) (22). This range of cell types indicates the general applicability of the assay.

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