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Invited Review

Quantitative *in situ* hybridization for the evaluation of gene expression in asynchronous and synchronized cell cultures and in tissue sections

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Summary. We describe an image analysis (IA) system that has been applied for the quantitative evaluation of mRNAs evidenced by *in situ* hybridization (ISH) with radiolabelled probes in cultured cells and in tissue sections. The ISH-IA method was used for the evaluation of cultured cell morphological parameters such as cell and nucleous area (CA and NA, respectively) in parallel with the levels of mRNAs detected as hybridization grains areas (GA). The evaluation of these parameters, together with the analysis of the levels of mRNAs (*c-jun*, cyclin A) specific for given cell cycle phases (i.e. G1 and S/G2), allowed the identification, in asynchronous cultures of human skin fibroblasts, of cells in G1 and S/G2 phases. The mRNA levels measured by ISH-AI were comparable with those detected by RT-PCR. This method was also applied for the analysis of fibronectin (FN) gene expression in control skin fibroblasts in relationship with the different phases of the cell cycle and in comparison with a tumor cell line (Sk-Hep1), heterogeneous either for morphometric parameters or for the levels of this transcript. Finally, the ISH-AI was applied for the semiquantitative evaluation of the expression, localization and alternative splicing pattern of FN mRNA in normal liver and in hepatocellular carcinoma (HCC) tissue sections.

Key words: Cell cycle, Fibronectin, Gene expression, Hepatocellular carcinoma, *In situ* hybridization

Introduction

The study of gene expression on mRNAs purified from tissues and *in vitro* cultured cells by filter hybridization with specific probes (i.e. Northern blotting and dot-blot) or by RT-PCR, allows the evaluation of mRNA levels representing the average value of molecules in the cell population under study. mRNA levels are often nonuniform and can vary as a function of differentiation stage, cell cycle, selection, etc.

The *in situ* hybridization (ISH) (Buongiorno-Nardelli and Amaldi, 1969; Gall and Pardue, 1969; John et al., 1969) overcomes these problems since it enables the identification and the visualization of mRNAs on target tissues and *in vitro* cultured cells, so that the nucleic acid can be localized in association with given cell types expressing specific mRNAs.

Until now, ISH has been mainly used as a qualitative tool for the detection of infectious agent genome or for the study of gene expression (Brahic and Haase, 1978; Haase et al., 1984; Lawrence and Singer, 1986; Sandberg and Vuorio, 1987; Basset et al., 1990; Pyke et al., 1991; McNicol and Farquharson, 1997), using either radiolabelled or non-isotopic reporter molecules. Quantification of autoradiograms by computer-assisted image analysis (IA), measuring diffuse integrated optical density, has been developed by Davenport and Nunez (1990) and by Moro et al. (1990).

We have applied ISH with radiolabelled probes in combination with a computer-assisted image analysis (IA) system giving, in an interactive mode, the measure of the autoradiographic grain areas and the measure of morphometric parameters in cultured cells and in tissue sections (Moro et al., 1990, 1992; Colombi et al., 1993). In particular, we have applied ISH-IA for the study of the expression of fibronectin (FN) mRNA in: 1) tumor cell lines expressing homogenous or heterogenous levels of this transcript (Moro et al., 1990); 2) skin fibroblasts derived from Ehlers-Danlos syndrome (EDS) (Pope and Burrows, 1997) patients showing an altered FN mRNA splicing at the EDA region (Colombi et al., 1991); and 3) human laryngeal and ectocervical carcinomas in relationship with tumor grading (Moro et al., 1992; Barlati et al., 1994, 1995). Finally, ISH-IA was applied for the study of the modulation of the levels of FN, collagen type I (proα1) and their integrin receptor (i.e.

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α5, α2 and ß1) mRNAs by dexamethasone in control and EDS skin fibroblasts (Zoppi et al.,1998).

Here we report the application of ISH-IA for the quantitative evaluation of gene expression in: 1) asynchronous and synchronized cultured human skin fibroblasts; 2) a human heterogeneous hepatoma-derived cell line; and 3) tissue sections from human normal and tumor liver histological sections. We demonstrate that this quantitative procedure is a powerful tool for the analysis of gene expression. In particular, ISH-IA is an invaluable tool when a limited number of cells is available and when gene expression has to be evaluated in association with specific cell phenotypes detectable in cell-cycle phases and in differentiation stages.

Results and Discussion

Evaluation of cell-cycle-related gene expression in asynchronous and synchronized dermal fibroblasts

Cell synchronization

The ISH-AI method was applied for the study of gene expression in a human control skin fibroblast culture in relationship with the different phases of the cell cycle.

For this purpose, we characterized the AB2 fibroblast culture obtained in our lab (8th *in vitro* passage) for the time of synchronization in G0 phase, by starvation in serum-free medium, as previously reported (De Petro et al., 1994). During this treatment, the cells were monitored at following times for the presence of mitosis (Mitotic Index M.I.) by optical microscopy and by incorporation of 5-bromo-2'deoxyuridine (BrdU) (Boehringer Mannheim Biochemica, Germany) into the replicating DNA (Girard et al., 1991). In particular, the analysis of the incorporated BrdU in the cell nuclei, by immunofluorescence microscopy with an anti-BrdU monoclonal antibody, allowed the determination of the percentage of the replicating cells (S phase) (Labelling Index: L.I.), in the culture under study during the starvation kinetic, in order to determine the time of starvation (time at which the M.I and the L.I. were equal to zero) required for synchronizing 100% of the cells in the G0 phase. For the AB2 fibroblast strain this time was found to be equal to 65 hours.

In order to induce the resting cells to enter the cell cycle, the G0-synchronized cells were supplemented with medium containing 10% fetal calf serum (De Petro et al., 1994). In the cycling cells the BrdU incorporation was followed and the L.I. was determined at increasing times after serum addition (Fig. 1). The evaluation of the L.I. allowed the identification of G1-synchronized cells between 0 and 12 hours) and of cells in G2/S phase (after 12 hours serum induction). The cells, in the different phases of the cell cycle, were fixed on slides for ISH with radiolabelled probes and were in parallel collected for RNA extraction and RT-PCR analysis. Not synchronized fibroblasts cultures were also grown and submitted to the same analysis procedure.

IA and ISH for the evaluation of morphometric parameters and gene expression in single cells

The cells synchronized and not synchronized in the different phases of the cell cycle were fixed on sterile microscope slides and hybridized with radiolabelled cDNA probes as previously reported (Moro et al., 1990, 1993, Colombi et al., 1993) specific for human *c-jun*, cyclin A (CycA) and fibronectin (FN) mRNAs. While *c-jun* mRNA is mainly expressed in G1 (Ryseck et al., 1988), CycA transcription is activated in S/G2 transition

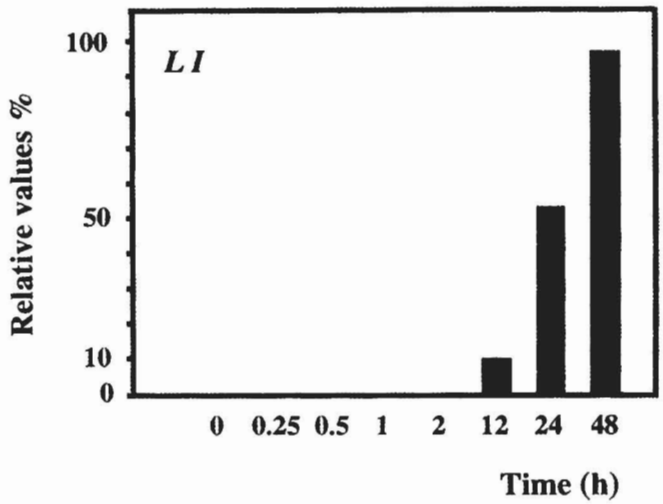

Fig. 1. Percentage of human skin fibroblasts (AB2) incorporating BrdU (Labelling index: LI) submitted to serum deprivation (time 0) and followed at different times after serum addition into the culture medium.

Table 1. Quantitative evaluation by IA of the Cell Area (CA) and of the Nucleous Area (NA) in synchronized (G0, G1 and S/G2) and not synchronized (NS) cultured skin fibroblasts after ISH.

		CA[a]	NA[a]
G0	x	29247	3649
	σ	5459	397
	CV	19	11
G1	x	30428	4006
	σ	3557	475
	CV	12	12
S/G2	x	47558	6036
	σ	7003	901
	CV	15	15
NS	x	33732	4603
	σ	9098	1177
	CV	28	26

[a]: CA and NA averages (x) out of 150 cells for each cell group; σ: standard deviation; CV: coefficient of variation. G0: the cells were synchronized by serum starvation; G1: G0-synchronized cells were cultures for 2 hours in complete medium; S/G2: G0-synchronized cells were cultured for 16 hours in complete medium; NS: not sinchronized cells.

(Girard et al., 1991) and FN mRNA is constitutively expressed by *in vitro*-grown skin fibroblasts (Colombi et al., 1993).

By image analysis (IA) with the Magiscan Image Analysis System (Joyce Loebl, Gateshead, England) using a task-list file developed from IN SITU software, available on the Magiscan, we performed in a semiautomatic mode the quantitative evaluation of the autoradiographic signals associated with single cells and evaluated for every cell the cell area (CA) and the nucleous area (NA) (Colombi et al., 1993). Cells in mitosis were not analyzed.

The IA on 150 cells, synchronized in the different phases of the cell cycle as reported above, showed that while the CA and the NA in G1-cells only slightly increased, if compared with resting G0-cells, these parameters increased, on average, by about 1.6-folds in S/G2-cells (Table 1). An approximate doubling of the CA and of the NA is expected in a proliferating cell which replicates its DNA and the cellular apparata before mitosis. In the cell population under study the IA showed an increase in these parameters which was lower than 2: this can be ascribed to the analysis of the cell surfaces instead of the volumes. The same parameters were also evaluated in not synchronized cells; Table 1 reports the average values obtained for the different phases of the cell cycle, with the exclusion of cells in mitosis. When the cell parameters (CA and the NA) of asynchronous cultures were ordered on the basis of the data obtained from the synchronized cell cultures, the two distributions overlapped. We concluded that the quantitative evaluation of the CA and the NA by IA on fixed cells also allows the identification of cells in G1 and in S/G2 phase in a not synchronized cell population.

The distribution of the not synchronized cells analyzed by ISH-AI is representative of the living cell population for the different phases of the cell cycle, as demonstrated by the comparison of the distribution obtained by plotting the number of cells analyzed as a function of the NA (Fig. 2b) and the distribution

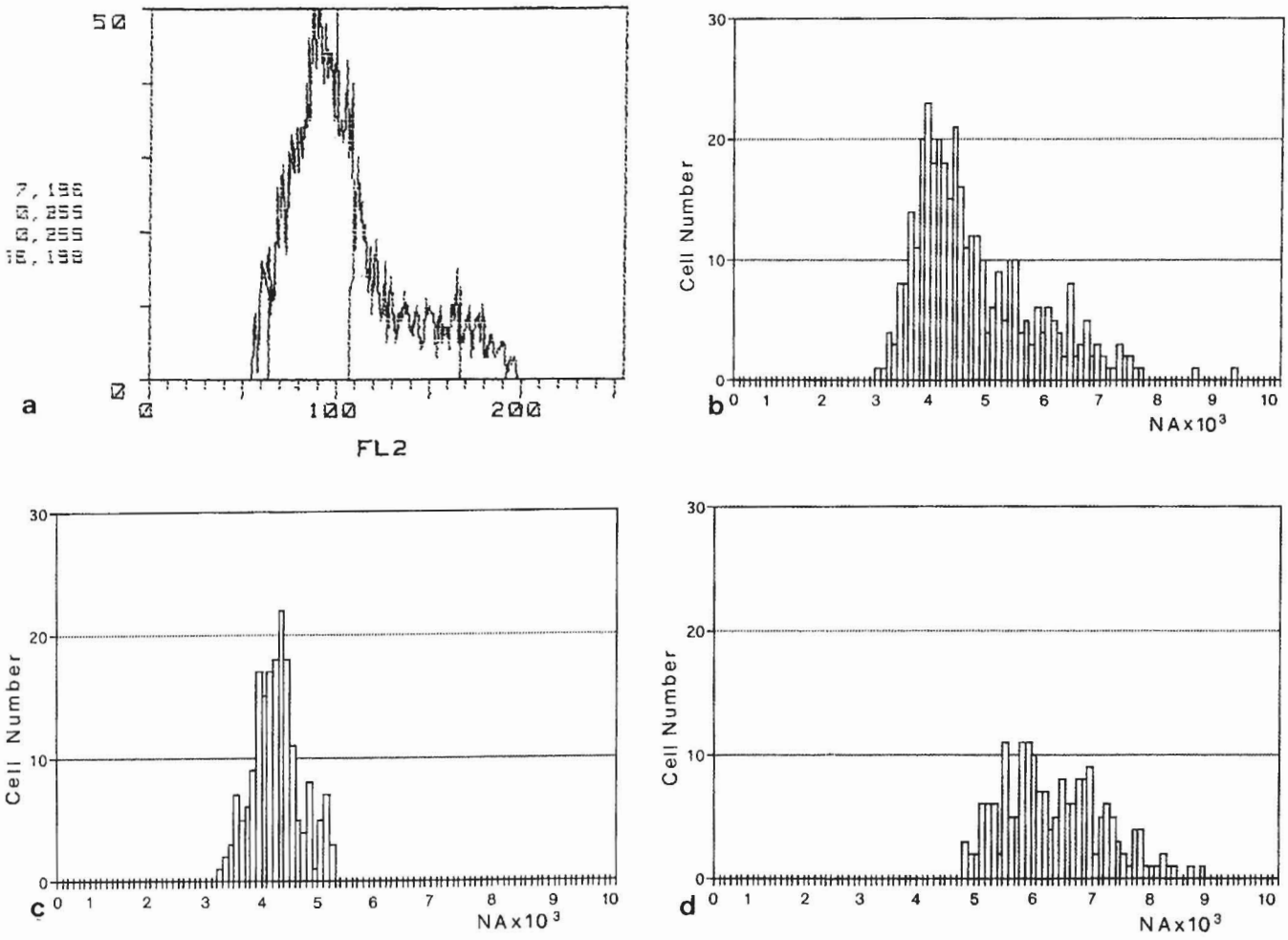

Fig. 2. Comparison of the DNA content (FL2) **(a)** and of the nucleous area (NA) **(b, c, d)** in asynchronous (a, b) and synchronized G1 (c) and S/G2 (d) *in vitro* cultured human skin fibroblasts obtained by flow cytometry (a) after incorporation into the DNA of propidium iodide, and by IA after ISH (b, c, d).

obtained on the same cell population by flow cytometry (Fig. 2a), after incorporation into the DNA of propidium iodide. The distributions of cells synchronized in G1 (Fig. 2c) and in S/G2 phases (Fig. 2d) taken together overlap with that of asynchronous cells (Fig. 2b).

The evaluation of the levels of cell-cycle-related transcripts, i.e. the *c-jun* and CycA mRNA in the synchronized cells, by IA on cells *in situ* hybridized with specific probes, showed that the expression of *c-jun* gene is activated, as expected, in G1 cells and this mRNA undergoes a rapid decrease after this phase of the cell cycle (Table II and Fig. 3A). A cell-cycle phase-specific activation was also observed for CycA gene: as expected, the CycA mRNA expression was at the highest level in S/G2 transiting cells while it was detected at very low levels in G0 and G1 cells (Table 2 and Fig. 3B).

We also analyzed the expression of FN gene, which should be costitutively activated in *in vitro* cultured skin fibroblasts. Table 2 and Fig. 4 (A and B) show that FN mRNA was costitutively expressed in cycling cells; in particular, a high level of this mRNA was evaluated in G0 resting cells and its amount increased in G1 and in G2 cells: a 1.8-folds GA (Grains Area) increase was observed in S/G2 cells, if compared with G1 cells. Since the increase of this mRNA parallels the increase by about 1.7-folds of the CA (Cell Area) measured in these cells (Table 1), these data indicate that FN mRNA is expressed in skin fibroblasts as a function of the cell surface, as previously reported for another skin fibroblast strain (Colombi et al., 1993). The parallel increase of the GA and of the CA is also evidenced in Table 2 by the FR (Fraction Region) (GA/CA) which is constant in the different cell cycle phases. These data underline that FN, an adhesive protein, must be continuously synthesized by skin fibroblasts, in order to maintain its adherence to the substrate, and that its level must be proportional to the increase in the cell surface (from G1 to G2). Since a linear correlation exists between the cellular (not shown) and the nuclear area (Fig. 5) and the levels of FN mRNA expressed as GA, and since the control of the expression of FN is mainly at the transcription level (Colombi et al., 1986), the level of FN mRNA expressed by the cells under study must be a function of gene dosage. Following DNA duplication, FN genes are actively transcribed at the same rate and therefore cells in S/G2 phase (4 FN genes) produce approximately twice the amount of FN mRNA of G1 cells (2 FN genes).

The levels of the *c-jun* , CycA and FN mRNAs were also evaluated by ISH-AI in not asynchronous skin fibroblasts. Table 2 shows that in these cells the levels of the different mRNA species expressed as GAs correspond to the averages of the values measured in cells in the different phases of the cell cycle. These values, considered independently from the CA and the NA,

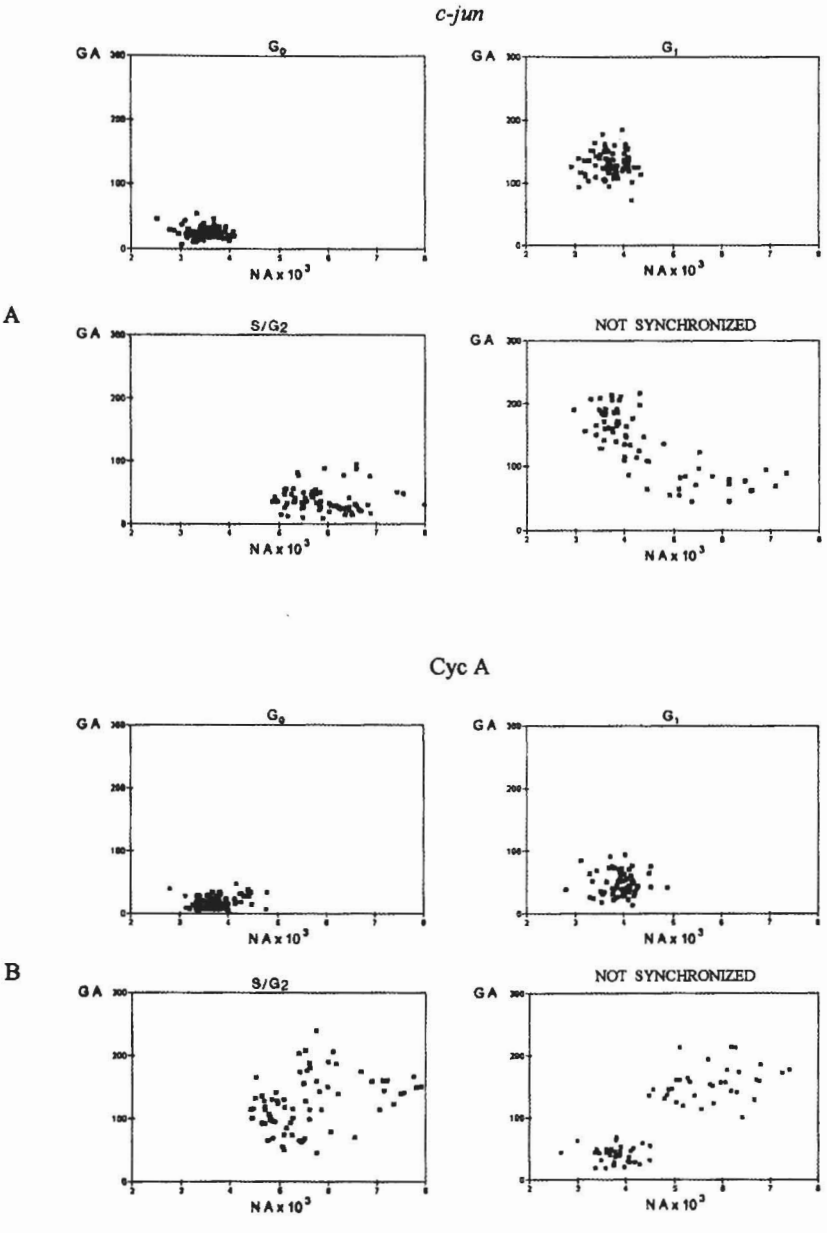

Fig. 3. Level of cell-cycle-related *c-jun* **(A)** and CycA **(B)** mRNAs (GA) expressed in control skin fibroblasts synchronized by serum starvation in G0 and, after serum addition, in G1 and S/G2 phases evidenced by ISH and quantitatively evaluated by IA. The GAs are reported as a function of the NA.

therefore, do not give information about the expression of specific mRNAs in relationship with cell cycle phases.

Figs. 3A,B, 4a show the distribution of the GAs evaluated after ISH-AI with the different probes as a function of the NA either in synchronized or in not synchronized cells. The distributions obtained for the not synchronized cells clearly overlap with the combination of the distributions obtained with the same probe in synchronized G1 and S/G2 cells. Therefore, it is possible to identify in the not synchronized cell population the presence of G1 (average NA of about 4000 pixels) and S/G2 (NA of about 6000 pixels) cells and evaluate the respective GAs. Clearly, resting G0 cells are not present in proliferating cell cultures and can be observed only after serum starvation.

This evidence clearly shows that ISH-AI allows the qualitative and quantitative evaluation of cell-cycle-related gene expression in not synchronized cell populations, when combined with the evaluation of the NA and of the CA. This is an invaluable tool since it requires a small number of fixed cells (less than 500

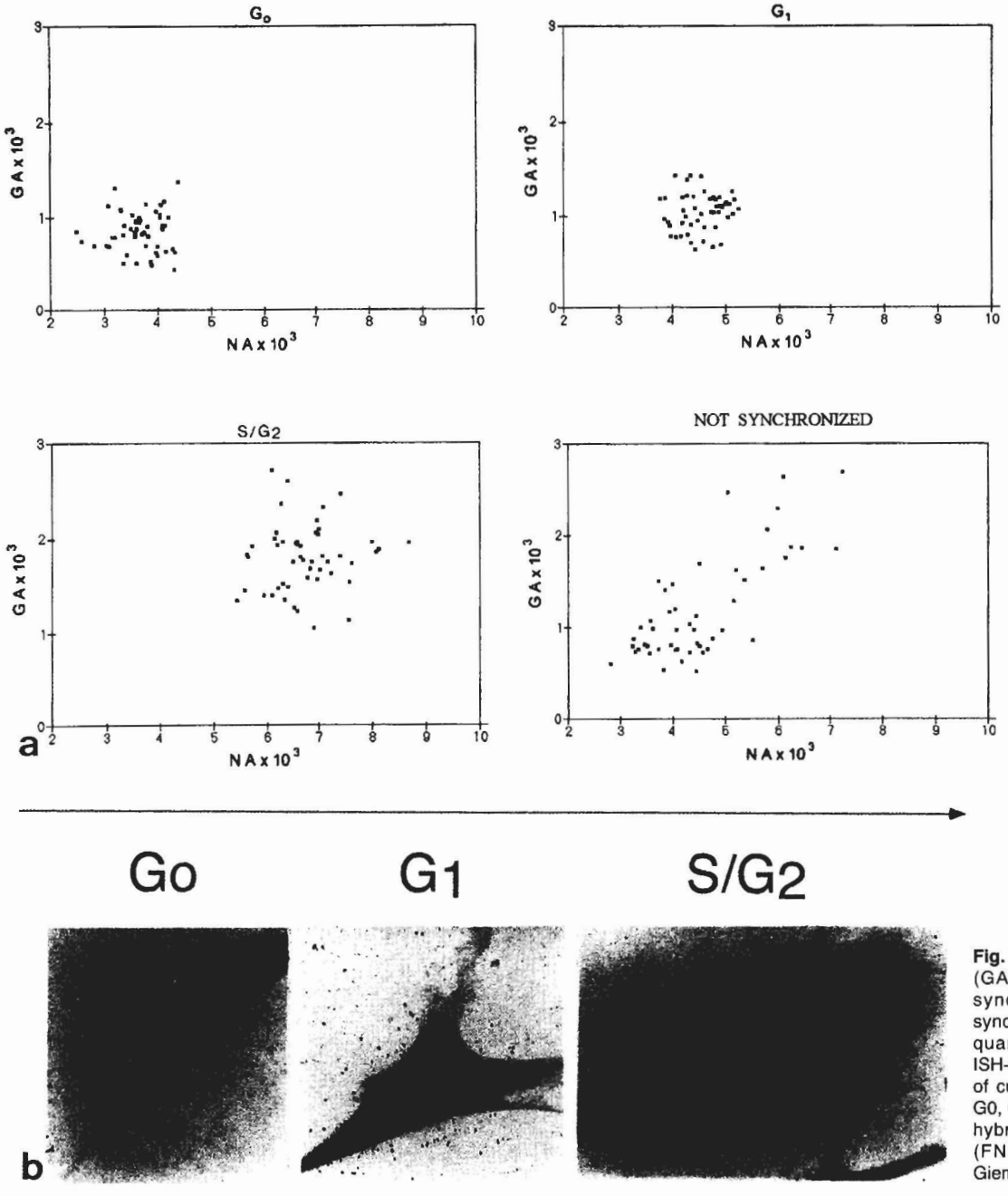

Fig. 4. a. Levels of FN mRNA (GA) expressed in control synchronized and not synchronized skin fibroblasts quantitatively evaluated by ISH-IA. **b.** Optical microscopy of cultured skin fibroblasts in G0, G1 and S/G1 phases after hybridization with FN cDNA (FNI-1) and staining with Giemsa. x 630

cells) which can be analyzed in the main phases of cell cycle, without synchronization's procedure.

Evaluation of cell-cycle-related gene expression by comparative RT-PCR: comparison with ISH-AI

On the synchronized and not synchronized skin fibroblast cultures we also performed the analysis of the expression of *c-jun*, CycA and FN mRNAs by RT-PCR, in order to compare this method with the ISH-AI. For this purpose, total RNAs were purified from the skin fibroblasts strain cultured in complete medium and synchronized in the different phases of the cell cycle, as described above. Similar amounts of RNAs were retrotranscribed and amplified with pairs of oligonucleotides specific for *c-jun*, CycA, FN and glucose-6 phosphate dehydrogenase (GAPDH, a house keeping-like enzyme) cDNAs (Fig. 6A,B). In not synchronized cultures (NS), heterogeneous for the presence of cells in the different phases of the cell cycle, all genes were expressed. The levels of FN mRNA corresponded to the average of the levels expressed in cells in the different phases of the cell cycle. In synchronized G0 cells *c-jun* and CycA genes were not transcribed, while GAPDH and FN genes were expressed. In cells grown after starvation in complete medium, we observed the early activation of *c-jun* gene starting from 15 minutes after 10% medium addition (G1 phase). After 12 hours, the levels of *c-jun* mRNA detected were reduced to less than 10% of those measured in G1 phase. CycA mRNA was not expressed in G1-cells while it was present at high levels in S/G2-cells. FN and GAPDH mRNAs were constitutively expressed by the cells transiting from G1 to S/G2 phase.

The RT-PCR method here reported allows, as well as ISH-IA, the evaluation of cell cycle phase-specific genes. However, for obtaining this information, the following is needed: 1) the characterization of the cell culture under study for the time requested for the G0-synchronization, after serum starvation; 2) the definition of the cell cycle timing; 3) the preparation of separate cell cultures to be submitted to RNA extraction at the different steps of the cell cycle after synchronization; 4) RNA purification, and evaluation of its concentration in each sample; 5) RT-PCR for the transcript under study and for an house keeping-like mRNA (GAPDH), in order to allow a comparative and semiquantitative evaluation of the mRNAs; and 6) electrophoresis and quantitative evaluation of the RT-PCR products by IA.

In spite of the complexity of ISH procedure, once it has been standardized, it allows the evaluation of cell-cycle-related gene expression on a small number of asynchronous cells which only need to be fixed on the slide. The combination of ISH with IA and the evaluation of cell morphometric parameters overcome the cell synchronization procedure and allows the evaluation of the levels of transcripts expressed by cells in any phase of the cell cycle. Moreover, ISH-IA analysis can be particularly useful as compared with semiquantitative/comparative RT-PCR, since it also allows the simultaneous observation of mRNAs expressed at variable levels in heterogeneous cell population for poorly transcribed genes. Finally, we have reported that ISH-IA, in combination with dot-blot analysis, allows the detection of the approximate number of molecules of given mRNA species in cultured cells (Moro et al., 1990). This approach is very sensitive since it allows the detection of illegitimate transcription products, as in the case of HeLa cell line which poorly

Table. 2. Levels of *c-jun*, CycA and FN mRNAs (Grains Area: GA) expressed by synchronized and not synchronized (NS) skin fibroblasts evaluated by ISH-IA.

		c-jun[a]		CycA[b]		FN[c]	
		GA[d]	FR[e]	GA	FR	GA	FR
G0	x	25	0.08	19	0.06	825	2.8
	σ	9		10		212	
	CV	37		50		26	
G1	x	129	0.42	47	0.15	1018	3.3
	σ	20		18		193	
	CV	16		39		20	
S/G2	x	36	0.08	126	0.26	1796	3.8
	σ	19		43		360	
	CV	54		34		20	
NS	x	137	0.41	98	0.29	1188	3.5
	σ	51		61		563	
	CV	37		62		47	

[a]: the probe used was a 325 bp *c-jun* cDNA, obtained by RT-PCR standard procedure on total RNA from control fibroblasts with the primers 5'-dGGAAACGACCTTCTATGACGATGCCCTCAAA-3' and 5'r-GAACCCCTCCTGCTCATCTGTCACGTTCTT-3' (Angel et al., 1988). [b]: the probe used was a 227 bp CycA cDNA, obtained as reported above with the primers 5'd-GCTCCAAGAGGACCAGGAGAATAT-3' and 5'rTGGTGAACGCAGGCTGTTTACTGT-3' (Wang et al., 1990). [c]: the probe used was a 2.1 kb FN cDNA fragment obtained from the pFH1 plasmid (Kornblihtt et al., 1985). [d]: GA average (x) out of 150 cells per each group. [e]: FR (GA/CA) (x10^2) averages (x) obtained out of 150 cells for each group. s: standard deviation. CV: coefficient of variation.

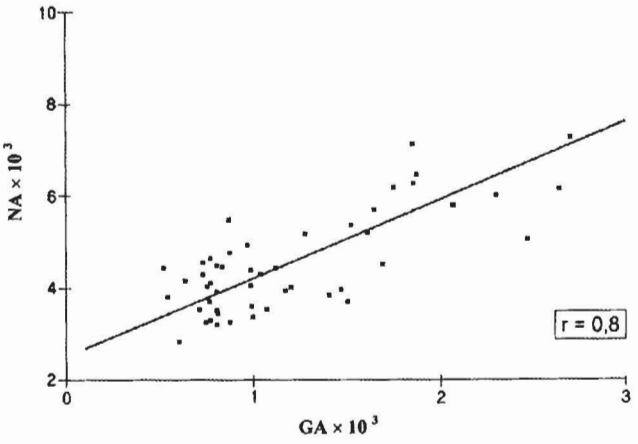

Fig. 5. Correlation between the levels of FN mRNA (GA) expressed by human *in vitro* cultured skin fibroblasts, measured by ISH-IA, and the nucleous area (NA).

expresses FN mRNA (2-4 molecules per cell) (Moro et al., 1990).

Quantitative evaluation of mRNA in a hetero-geneous cell population by ISH-IA

ISH was performed on control skin fibroblasts (Flow7000) (Fig. 7a) and on a cell line derived from a human hepatoma (Sk-Hep1) (Presta et al., 1986) (Fig. 7b) using a radiolabelled FN cDNA (FNI-1) as probe. While control cells expressed FN mRNA homogeneously, Sk-Hep1 cells expressed this mRNA in a heterogeneous fashion and two subpopulations (Sk-Hep1C2 and Sk-Hep1C3) were evidenced either on the basis of the expression of FN mRNA or of the morphology. The Sk-Hep1C3 subpopulation (7.8% of the total cells determined out of 700 cells) was composed of large fibroblastoid cells expressing levels of FN mRNA 50% higher than control fibroblasts and 11.2 higher than the Sk-Hep1C2 subpopulation (92.2% of the total cells) (Table 3). The Sk-Hep1C2 cells were round-shaped and showed a CA 1.8-folds lower than the Sk-Hep1C3 cells. Both cell subpopulations maintained their morphological and expression patterns after *in vitro* cloning. On these cells, ISH-IA combined with quantitative mRNA dot-blot analysis allowed the estimate of the approximate number of mRNA molecules present in single cells (Table 3) (Moro et al., 1990).

Using ISH-IA, we have also observed the presence of heterogeneity in a human rhabdomyosarcoma-derived cell line (RD) (Colombi et al., 1993). Therefore, this approach can be applied to the search of different subpopulations, defined by the levels of given mRNA species, in cells derived from control or pathological tissues and/or from *in vitro* cultured cells, after treatments with chemicals, drugs, or transforming agents.

ISH-IA on tissue sections

ISH-IA was also applied for the study of FN mRNA

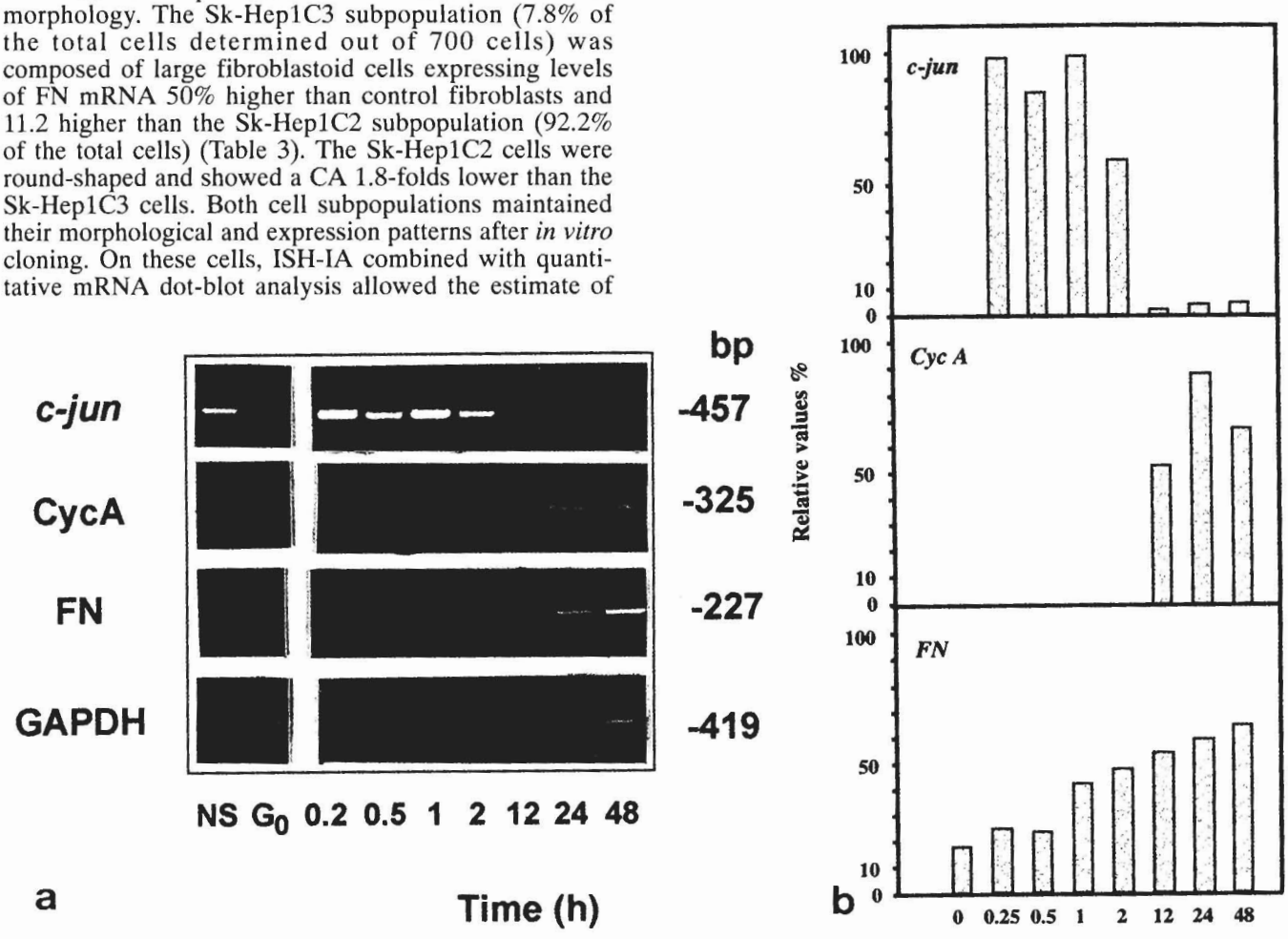

Fig. 6. Evaluation of c-jun, CycA and FN mRNAs by semiquantitative RT-PCR on total RNAs purified from not synchronized (NS) and synchronized control skin fibroblasts. **a:** agarose gel electrophoresis of the RT-PCR products obtained with the following primers sets: *c-jun* - 5'dGGAAACGACCTTC TATGACGATGCCCTCAA3', 5'rGAACCCCTCCTGCTCATCTGTCACGTTCTT3' (Angel et al., 1988); CycA - 5'dGCTCCAAGAGGA CCAGGAGAATAT3', 5'rTGGTGAACGCAGGCTGTTTACTGT3' (Wang et al.,1990); FN - 5'dGCCTGGTACAGAATATGTAGTG3', 5'rATCC-CAGCTGATCAGTAGGCTGGTG3' (Gardella et al., 1993); GAPDH - 5'dAAGAAGATGCGGCTGACTGTCGAGCCACAT3', 5'rTCTCATGGT TCACACCCATGACGAACATG3' (Irving et al., 1992). For *c-jun* cDNA were performed 26 cycles as follows: 30" at 94 °C, 30" at 62 °C and 60" at 72 °C. For CycA cDNA were performed 24 cycles as follows: 30" at 94 °C, 25" at 58 °C and 45" at 72 °C. For FN cDNA were performed 23 cycles as follows: 30" at 94 °C, 25" at 58 °C, 45" at 72 °C. For GAPDH cDNA were performed 25 cycles as follows: 30" at 94 °C, 30" at 60 °C and 30" at 7 °C. **b**: The electrophoretograms in **a** were quantitatively evaluated by IA and expressed as integrated densities (ID). The ID obtained for *c-jun*, CycA and FN mRNAs were normalized to the levels of the GAPDH house keeping-like gene and the highest value measured was considered equal to 100%.

expression in histological tissue sections obtained from human liver and hepatocellulat carcinoma (HCC).

We have previously reported the analysis by comparative RT-PCR of the levels of two FN mRNA isoforms, derived from alternative splicing of the EDA and EDB regions (Kornblihtt et al., 1985), in histological sections from HCC, peritumoral tissue and distal, unaffected, liver. While EDA$^+$ and EDB$^+$ mRNAs were not present in control liver and in non-tumorous liver tissue, they were always (14 cases out of 14) expressed by HCCs (Tavian et al., 1994). Therefore, EDA$^+$ and EDB$^+$ FN mRNA isoforms are molecular markers for HCC.

ISH-IA on peritumoral liver and HCC sections performed with two FN radiolabelled cDNA probes, the III-9 recognizing all FN mRNA isoforms (Gardella et al., 1993) and the EDA$^+$, hybridizing only with FN mRNA containing the EDA region (Colombi et al., 1991), showed levels of FN mRNA in peritumoral tissue which were lower than those detected in the HCC sections (Fig. 8). FN mRNA levels were increased as a consequence of the enhanced expression of the EDA$^+$ isoform (Table 4). The increase of FN EDA$^+$ mRNA levels in HCC was homogenous in all tumor cells.

The semiquantitative analysis by ISH-IA gave evidence similar to those obtained by RT-PCR on the same specimens, although ISH-IA also allowed the semiquantitative evaluation of the transcripts in relationship with specific cell types. The ISH-IA method could be more informative in cases of tissues heterogeneously expressing a given mRNA species or in cases in which there is a modulation of the mRNA levels in relationship with tumor grading, as we observed in human laryngeal and ectocervical carcinomas (Moro et al., 1992; Barlati et al., 1995).

Fig. 7. ISH-IA on cell cultures expressing homogeneous levels (Flow7000, control fibroblasts) **(a)** and heterogeneous levels (Sk-Hep1, hepatoma cell line) **(b)** of FN mRNA. Optical microscopy after hybridization with the FNI-1 2.1 cDNA (Kornblihtt et al., 1985) radiolabelled probe and after staining with Giemsa. x 630

Conclusions

The ISH-IA method here reported is an invaluable tool for the analysis of gene expression in asynchronous *in vitro* cultured not tumorigenic diploid cells, since, evaluating a limited number of cells, it allows the quantitation of mRNA species in relationship with the different phases of the cell-cycle, avoiding the cell synchronization procedures.

It should be pointed out that the ISH-IA cannot be applied for the evaluation of cell-cycle-related genes in polyploid (tumor) cells. This approach also allows the identification of cell subpopulations present in a given cell line. In combination with dot-blot analysis, ISH-IA allows the evaluation of the approximate number of molecules of a given mRNA expressed in *in vitro* cultured cells. ISH-IA can also be applied for the quantitative evaluation of mRNA isoforms generated by alternative splicing processes which are expressed in a

Table 3. Quantitative evaluation of FN mRNA levels in a normal fibroblast strain (Flow7000), in a hepatoma heterogeneous cell line (Sk-Hep1) and in two Sk-Hep1 clones (Sk-Hep1C2, Sk-Hep1C3) performed by ISH with a 2.1 kb radiolabelled FN cDNA (FNI-1) and by IA. IA was performed on 50 cells per each type.

CELL TYPE		GA	CA	NA	FR	Mol/Cell
Flow7000	x	1270	34000	3800	3.7	115±32
	σ	503	7746	770		
	CV	40	23	20		
Sk-Hep1	x	1388	33651	7740	4.1	n.d.
	σ	1356	10848	2626		
	CV	98	32	34		
Sk-Hep1C2	x	226	24079	7453	0.9	17±7
	σ	70	5401	1519		
	CV	31	22	20		
Sk-Hep1C3	x	2550	43222	8575	5.9	209±89
	σ	869	2564	2317		
	CV	34	6	27		

GA: grain area; CA: cell area; NA: nucleous area; FR: fraction region ($GA/CAx10^2$); x: average out of 50 cells; σ: standard deviation; CV: coefficient of variation; Mol/cell: number of FN mRNA molecules per cell, as evaluated by comparison of ISH-IA with dot-blot analysis (Moro et al., 1990). n.d.: not determined.

Table 4. Semiquantitative evaluation of FN mRNA isoforms in histological sections from an HCC bearing patient biopsies (peritumor and tumor tissues) by ISH-AI with radiolabelled FN cDNAs recognizing all FN mRNA species (III9, 419 bp) and EDA^+ species (EDA^+, 184 bp).

		TOTAL FN mRNAs GA[a]	EDA^+ FN mRNAs GA[a]	(%) EDA^+/Total
Peritumor	x	945.0	510.0	54
	σ	491.3	139.3	
	CV	35.0	16.6	
Tumor	x	2495.0	2070	83
	σ	285.3	536.9	
	CV	7.6	16.5	

[a]: grains areas (GAs) were evaluated per each homogeneous tissue sample in 10 randomly chosen fields with the average area of 251924 pixels ± 1. The III9 FN cDNA probe was obtained by PCR on pFH111 (Kornblihtt et al., 1985) with the oligos 5'-dGCCTGGTACAGAATATG TAGTG3' and 5'-rATCCCAGCTGATCAGTAGGCTGGTG3' as reported by Tavian et al. (1994); the EDA probe was obtained by PCR on pFH111 DNA with the oligos: 5'-dCACTGATGTGGATGTCGATTCC3' and 5'-rCACTGACTGTGTACTCAGAACC3'

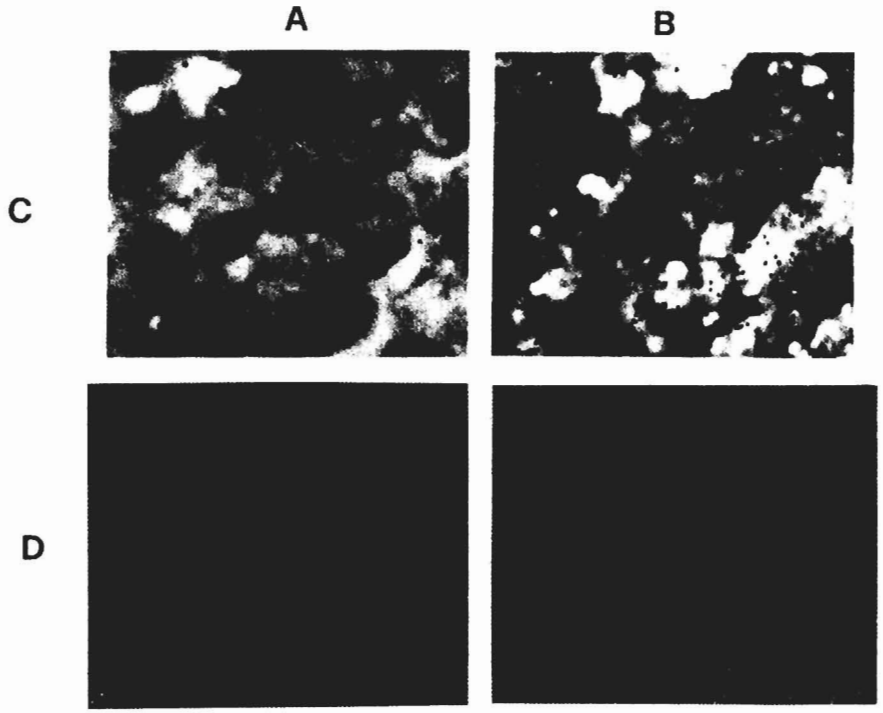

Fig. 8. FN total mRNAs detected by ISH-IA in histological sections from human peritumoral hepatic tissue **(A)** and HCC **(B)**, detected by optical microscopy after ISH and Giemsa staining **(C)** and after IA of the ISH **(D)**. The radiolabelled probe used was the FN III-9 cDNA. x 630

given cultured cell population. Finally, ISH-IA is the method of choice for the identification in histological sections of the cell types expressing a given mRNA or its isoforms and for the semiquantitative evaluation of mRNAs modulated in relationship with different physiological and pathological conditions.

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