Methodologies for specific intron and exon RNA localization in cultured cells by haptenized and fluorochromized probes

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SUMMARY

We have determined optimal conditions for the detection of mRNA sequences in cultured cells by non-radioactive in situ hybridization. For this purpose a number of different cell lines have been used: rat 9G cells for the detection of human cytomegalovirus immediate early mRNA, and HeLa as well as 5637 carcinoma cells for the detection of housekeeping gene mRNAs. Extensive optimization of fixation and pretreatment conditions revealed that most intense hybridization signals are obtained when cells are grown on glass microscope slides, fixed with a mixture of formaldehyde and acetic acid, pretreated with pepsin and denatured prior to hybridization. In addition, we also studied the potential of fluorochromized probes for the direct detection of multiple RNA sequences. The optimized in situ hybridization procedure revealed that immediate early mRNA transcripts are, in addition to a cytoplasmic localization, localized within nuclei of rat 9G cells. Double hybridization experiments showed that intron and exon sequences colocalize within the main nuclear signal. In addition, the presence of small, intron-specific, fluorescent spots scattered around the main nuclear signals indicates that intron sequences which are spliced out can be visualized. Additional information about the functioning of cells could be obtained by the detection of mRNA simultaneously with bromodeoxyuridine, incorporated during S-phase, or its cognate protein.

The sensitivity of these methods is such that mRNAs of abundantly expressed housekeeping genes can be detected in a variety of cell lines with high signal to noise ratios.

Key words: in situ hybridization, mRNA, introns, BrdU
duced by immunochemicals; and third, penetration problems of antibodies are eliminated. DNA in situ hybridization studies revealed that fluoresceinated probes are about ten times less sensitive than indirect techniques but result in extremely low noise. This low noise characteristic of direct techniques can be employed to increase detection sensitivity by using integration of fluorescence signals with a cooled CCD camera (Wiegant et al., 1991a,b). Recently, fluoresceinated oligonucleotides have also been successfully used for the detection of abundantly expressed neuronpeptide mRNAs in the brain of Lymnaea stagnalis (Dirks et al., 1990, 1991).

Here we describe a protocol which allows the detection of the abundant human cytomegalovirus immediate early (HCMV IE) mRNA and the somewhat less abundant mRNAs of the housekeeping genes human elongation factor (HEF) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with high signal-to-noise ratios using haptenized and fluoresceinated probes. Moreover, the feasibility of mRNA in situ hybridization together with an immunocytochemical protein detection as well as the combination of in situ hybridization with the immunocytochemical detection of bromodeoxyuridine (BrdU) was investigated. Furthermore, our study to the nuclear localization of intron- and exon-specific sequences of IE mRNA in rat 9G cells (Raap et al., 1991) has been extended and its implications discussed.

MATERIALS AND METHODS

Cell lines

Rat 9G, HeLa and 5637 cells were cultured in Dulbecco’s minimal essential medium supplemented with 10% fetal calf serum but without phenol red at 37°C in a 5% CO2 atmosphere. For experiments on glass microscope object slides, cells were grown for two days in Petri dishes containing object slides which were un-coated or coated with either poly-L-lysine or gelatin chrome-alum. IE expression of rat 9G cells was induced by the addition of 50 µg/ml cycloheximide (Sigma) for 4 h (Boom et al., 1986). After incubation, the cells were rinsed in PBS and for the immunocytochemical detection of immediate early antigen (IEA), fresh medium was added and cells were cultured for an additional 16 h.

Fixation and pretreatment of cells

After culturing, cells were briefly rinsed in PBS and fixed in 4% formaldehyde, 5% acetic acid in 0.9% NaCl for 20 min at room temperature. A number of different fixatives were also evaluated. They include: methanol for 30 min; methanol/acetic acid (3:1, v/v) for 30 min; 75% ethanol, 23% acetic acid, 2% formaldehyde for 20 min; 4% and 1% formaldehyde; and 4% formaldehyde with 10% and 20% acetic acid for various times. After fixation, cells were rinsed in PBS for 5 min, dehydrated in ethanol and washed for 10 min in xylene or Histoclear at room temperature. Cells were then washed twice in 100% ethanol, rehydrated to PBS and treated with 0.1% pepsin (Sigma) in 0.01 M HCl for 10 min at 37°C. In order to inactivate pepsin and to preserve morphology, cells were rinsed in PBS and post-fixed in 1% formaldehyde in PBS for 5 min. Subsequently cells were rinsed in PBS and dehydrated in 70%, 90% and 100% ethanol. Variations in pretreatments are described in Results.

Probes and labeling

The following probes have been used for the detection of IE mRNA in rat 9G cells: the viral genomic pSS, containing the 5.0 kb Sphi-Sal1 fragment of the transfected IE region (Boom et al., 1986). A single-stranded RNA probe transcribed from exons-4-specific PCR product which was amplified using the primer 2581-2600 supplemented with phage T7 RNA polymerase promoter sequences. The RNA probe was fractionated by alkaline hydrolysis (Cox et al., 1984) to an average fragment length of 150 bases. A 40-mer oligonucleotide complementary to map position 1691-1730 was synthesized on an Applied Biosystems model 381. For the detection of intron-specific sequences of the IE mRNA induced rat 9G cells an 800 bp PCR probe was made with primer sequences 123-142 and 903-922. For the detection of exon-specific sequences a 939 bp exon-4-specific PCR probe was made with primer sequences 1661-1680 and 2581-2600 (Akrigg et al., 1985).

HEF and GAPDH mRNA were detected using plasmids containing, respectively, HEF-1 cDNA (Brands et al., 1986) and full-length GAPDH cDNA. 28 S rRNA was detected with a plasmid probe containing a 2.1 kb insert complementary to the 3’ site of human 28 S rRNA (Erickson et al., 1981; Bauman and Bentvelzen, 1988) and with a specific oligonucleotide probe complementary to position 2183-2202 (Chan et al., 1983).

Plasmid and PCR generated probes were labeled with digoxigenin-11-dUTP (Boehringer Mannheim FRG), biotin-11-dUTP (Sigma), fluorescein-11-dUTP (Amersham) fluorescein-12-dUTP (Boehringer Mannheim FRG), tetramethyl rhodamine-4-dUTP (Amersham), or coumarin-4-dUTP (Amersham/Boehringer) by nick translation. The exon-4-specific RNA probe was labeled during transcription with digoxigenin-UTP (Boehringer). Oligonucleotide probes were labeled with the fluoresceinated dUTPs or digoxigenin-11-dUTP using terminal transferase as described previously (Dirks et al., 1991).

In situ hybridization

Hybridization of plasmid and PCR probes was performed overnight at 37°C in 60% deionized formamide, 2×SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0), 50 mM sodium phosphate and 50 times excess of salmon sperm DNA under a coverslip. Probes were denatured at 80°C for 10 min and in some experiments probe and target sequences were denatured simultaneously under a coverslip for 3 min at 80°C. Final probe concentrations ranged from 1 to 5 ng/µl. For hybridizations with the RNA probe, the hybridization temperature was increased to 50°C. Double hybridizations were accomplished by mixing two different labeled probes in the same hybridization buffer with final probe concentrations of 5 ng/µl. After hybridization, cells were rinsed two times, 10 min each, in 60% formamide, 2×SSC at 42°C followed by two washes at room temperature for 20 min. Hybridizations with RNA probes were followed by three 10 min washes in 50% formamide at 60°C. Synthetic oligonucleotides were hybridized in 25% formamide, 2×SSC at room temperature and after hybridization slides were washed in 2×SSC, 3×10 min at room temperature. Finally, cells were rinsed in 0.1 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween-20 for 5 min.

Immunocytochemical detection

Digoxigenin was detected with a one-, a two- or a three-step procedure. The one-step procedure was accomplished with sheep anti-digoxigenin-FITC or -TRITC (Boehringer Mannheim) for fluorescent detection or sheep anti-digoxigenin conjugated to peroxidase (PO) or alkaline phosphatase (AP) for bright-field microscopy. Antibody dilutions were made in blocking medium
consisting of 0.1 M Tris-HCl, 0.15 M NaCl, 0.5% (w/v) blocking reagent (Boehringer Mannheim). In the two-step procedure, digoxigenin was detected with mouse monoclonal anti-digoxigenin (Boehringer Mannheim) and rabbit anti-mouse-FITC or -TRITC (Sigma). The three-step procedure was performed with monoclonal mouse anti-digoxigenin, sheep anti-mouse-digoxigenin and sheep anti-digoxigenin-FITC or -TRITC (all from Boehringer Mannheim). Peroxidase activity was visualized using 3,3′-diaminobenzidine (DAB, Sigma) and 0.01% H2O2 in 50 mM Tris-HCl, pH 7.4, containing 0.01 M imidazole. Alkaline phosphatase activity was detected in 0.16 mg/ml 5-bromo, 4-chloro-indoxyl phosphate and 0.33 mg/ml nitro blue tetrazolium salt in 0.2 M Tris-HCl, pH 9.2, 10 mM MgCl2.

Biotinated probes were detected by successively applying avidin-D-FITC or avidin-D-TRITC (Vector Laboratories, USA), biotinated goat anti-avidin-D (Vector) and another incubation with avidin-FITC or avidin-TRITC diluted in blocking reagent. All incubations were performed at room temperature for 30 min and after each incubation slides were rinsed 3×10 min in 0.1 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween (TBS).

Double hybridizations with two different haptenized probes were visualized by mixing anti-digoxigenin and avidin-TRITC in the first immunolayer. For the simultaneous detection of IE mRNA and IEG a mouse monoclonal anti-IEA (Biosoft) was combined with sheep antidigoxigenin-TRITC or avidin-TRITC in the first antibody step after hybridization. The second antibody step consisted of rabbit anti-mouse-FITC.

**Combined detection of IE mRNA and BrdU in rat 9G cells**

Rat 9G cells were induced for IE mRNA expression by adding cycloheximide to the culture medium. During the last 15 min of the cycloheximide treatment 10 μM BrdU was added to the medium. Cells were fixed as described and denatured prior to hybridization in 90% formamide, 2×SSC, pH 7.2 for 1 h at 70°C. After a short wash in ice-cold 70% ethanol, heat-denatured probe was added to the slides and the hybridization was performed overnight. Following post-hybridization washes slides were incubated with monoclonal anti-BrdU (IU4, Sanbio) and sheep antidigoxigenin-FITC for 1 h at 37°C, rinsed three times, 10 min, in TBS and incubated with rabbit anti-mouse TRITC. After three final washes, slides were embedded in an antifading solution.

**Microscopy and photography**

Fluorescent results were examined with a Leitz Dialux Epifluorescence microscope equipped with a 100 W mercury arc lamp and appropriate filter sets for red, green and blue fluorescence. For photography, 63× and 40× lenses with numerical apertures of 1.3 and a 640 ASA color slide film (3M) were used. Bright-field photomicrographs were taken on a 100 ASA color slide film (Konica R-100).

**RESULTS**

**Cell preparation, fixation and pretreatments**

In order to assess the optimal cell preparation method, rat 9G cells have been harvested by a variety of protocols. Cycloheximide-treated cells, grown in culture flasks, were harvested using a rubber policeman or by trypsinization. Cells were subsequently rinsed in PBS, collected in a pellet by centrifugation and either prepared for frozen sectioning or fixed in formaldehyde and embedded in paraffin. Alternatively, drops of cell suspensions were put on glass micro-

scope slides and cells were allowed to adhere to the glass. Frozen sections or intact cells were fixed in modified Carnoy’s (Dirks et al., 1990) or 4% formaldehyde for 15 min. Intact and sectioned cells were hybridized with digoxigenin-labeled pSs and hybrids were visualized by DAB staining. The hybridization results revealed positive hybridization signals in a subtraction of the cells, as expected, but the morphology of the cells was poor. In addition to a cytoplasmic signal, a number of cells also showed a nuclear, dot-shaped signal. When cell preparations were processed for fluorescence microscopy it was difficult to identify positive hybridization signals due to high autofluorescence. Searching for a better alternative to preserve the characteristic morphology of the rat fibroblasts, rat 9G cells were cultured directly on glass microscope slides which were un-coated, or coated with either gelatin chrome-alum or poly-L-lysine. Cells grown on glass slides showed more normal morphology and a minimum amount of autofluorescence. Furthermore, the use of coated slides resulted in a minimum loss of cells during the in situ hybridization procedure. However, coated slides were more susceptible to non-specific sticking of probe and antibodies.

The influence of different fixatives (see Materials and methods) on hybridization signals and morphology was investigated further. The best results were obtained when the cells were fixed in 4% formaldehyde, 5% acetic acid for 20 min and these results were consistent for all IE probes used in this study. Hybridization signals were clearly less intense when the cells were fixed in formaldehyde alone.

The beneficial effect of acetic acid in the formaldehyde fixative has also been investigated on the HeLa and 5637 carcinoma cell line using a 28 S rRNA probe. Cells were fixed in 4% formaldehyde containing 0%, 5%, 10% or 20% acetic acid for 1, 10, 20, 60 min and 16 h. For both cell lines, the best results with regard to signal strength and morphology were obtained when cells were fixed in 4% formaldehyde and 10% acetic acid for 20 min (Fig. 1A,B,C).

The same results were obtained for HEF mRNA detection in 5637 cells although there was only a small difference in signal intensity between cells fixed in formaldehyde containing 5 or 10% acetic acid. For the detection of HEF mRNA in HeLa cells, the presence of acetic acid in the fixative had only a minor influence on the hybridization signals.

In addition to proper fixation, it proved important to increase the accessibility of RNA sequences to the probe and antibodies. Without any pretreatment of the cells, a positive hybridization result could only be obtained with the rRNA probes, showing non-homogeneous staining of the cytoplasm and poor staining of nucleoli. In order to increase the accessibility of RNA target sequences, several treatments have been tested, including proteinase K and pepsin, rinses with xylene or Histoclear, freeze-thawing and denaturation. With all probes tested, the best and most consistent results were obtained when cells were treated with 0.1% pepsin for 10 min prior to hybridization. The influence of such pepsin treatment on the detection of HEF mRNA in HeLa cells is illustrated in Fig. 2A,B. As a result of the pepsin treatment, the morphology of the cells was
slightly affected, but further deterioration could be prevented by post-fixation with formaldehyde. Prolonged exposure to pepsin resulted in a dramatic loss of morphology and considerable cell loss. Treatment of cells with xylene or Histoclear alone had no effect on the hybridization result. Occasionally, however, when performed prior to pepsin treatment, we observed an increase in hybridization signal without an influence on the morphology of the cells. Freeze-thawing of cells prior to hybridization resulted also in positive hybridization signals, although less strong compared to hybridization signals of cells treated with pepsin. The morphology of the cells was, however, better preserved. Furthermore, hybridization signals of all probes were considerably improved by the simultaneous denaturation of probe and pepsin-treated cells as illustrated for the detection of HEF mRNA in HeLa cells (Fig. 2C).

Probes and detection systems

A variety of probe types have been employed for the detection of IE and housekeeping gene mRNAs as well as for
the detection of 28 S rRNA including plasmid, PCR-amplified DNA, RNA and synthetic oligonucleotide probes. For the direct in situ hybridization technique, probes were labeled with fluorochromes allowing their visualization after post-hybridization washes by fluorescence microscopy. Fluorescein- and rhodamine-labeled plasmid and PCR probes were successfully used for the detection of IE mRNA and 28 S rRNA. Weaker hybridization signals were obtained when probes were labeled with coumarin-derivatized dUTP. In HeLa cells, HEF mRNA could be visualized directly with rhodamine-labeled plasmid probe, although the signal was of low intensity (Fig. 3). We also examined synthetic oligonucleotides labeled at their 3’ ends with fluorescein and rhodamine. Strong hybridization signals were only obtained with fluorochromized oligonucleotides specific for 28 S rRNA. Fig. 4 shows a hybridization result obtained with HeLa cells and a rhodamine-labeled 28 S rRNA oligonucleotide. In contrast, the hybridization signals which were obtained with fluoresceinated IE mRNA specific oligonucleotides were hardly visible against background.

For the indirect detection of RNA sequences biotinated and digoxigenin-labeled probes have been used in combination with a number of different immunocytochemical detection systems in order to achieve optimal labeling intensities and low background staining. The strongest hybridization signals were obtained using the three-step immunology, which was therefore used as a standard detection method. In situ hybridizations detectable by fluorescence microscopy could also be visualized by bright-field microscopy using an AP or PO staining reaction. Fig. 5A,B shows the visualization of HEF mRNA and the centromeric regions of chromosome 1, to illustrate the specificity of the HEF probe hybridization and that accessibility was high.

IE, HEF and GAPDH mRNAs as well as 28 S rRNA have been successfully detected with nick-translated probes and no difference in sensitivity was observed between biotinated and digoxigenin-labeled probes. Essentially the same results were obtained with PCR probes, labeled with biotin-dUTP during the amplification reaction.

The strongest hybridization signals were obtained with biotin- or digoxigenin-labeled single-stranded RNA probes. A careful optimization of probe concentrations was, however, necessary in order to reduce non-specific hybridization to, presumably, rRNA sequences. The use of intact RNA probes resulted in a positive hybridization signal of cytoplasmic IE mRNA, but a nuclear hybridization signal was absent, whereas the use of fragmented RNA probes resulted in a cytoplasmic and nuclear hybridization signal.
Hybridization experiments with IE mRNA-specific oligonucleotides labeled with digoxigenin revealed a positive cytoplasmic staining in rat 9G cells, the intensity of which was low compared to that obtained with the other, larger, probe types used (data not shown). However, strong hybridization signals were obtained with digoxigenin-labeled oligonucleotides specific for 28 S rRNA in all cell types used. When fluorescence intensities were visually compared after hybridization, there was visually no difference between fluorescent or digoxigenin-labeled 28 S rRNA oligonucleotide and plasmid probes.

**Multiple RNA detection and combinations with proteins or BrdU**

The possibility of detecting different RNA sequences simultaneously using fluoresceinated probes was investigated in rat 9G cells. fluorescein-labeled plasmid 28 S rRNA probe and rhodamine-labeled pSS were mixed, hybridized, and examined directly after post-hybridization washes. All cells showed a green fluorescent signal and a number of these cells also showed a red fluorescent signal specific for IE mRNA as could be demonstrated by changing the appropriate filter combinations (Fig. 6). Both hybridization signals could also be observed simultaneously by use of a double band-pass filter (Omega) in which case the double-labeled cells showed a mixed colour.

Double-labeling experiments with intron- and exon-specific PCR probes, which were labeled with biotin and digoxigenin, allowed the simultaneous detection of pre- and mature IE mRNA sequences. The intron-specific signal, which was confined to the nucleus only, colocalized with the exon-specific nuclear signal (Fig. 7). Simultaneous detection of intron and exon sequences could also be achieved by combining a fluorochrome only a weak nuclear hybridization signal was observed.

The power of the in situ hybridization technique resides not only in the possibility of detecting different (m)RNAs simultaneously but also in the possibility of combining this technique with different immunocytochemical techniques. By combining the antibodies necessary for the detection of the hybrid and IEA it proved possible to visualize IE antigen and mRNA simultaneously without detectable decrease of the hybridization signal. As illustrated in Fig. 8 the combined detection of IE mRNA and protein revealed a heterogeneous staining pattern. Not all cells positive for IE mRNA showed IEA immunoreactivity and also, cells showing immunoreactivity for IEA did not always show IE mRNA hybridization. When the immunocytochemical detection of IEA was performed prior to hybridization a significant decrease in hybridization signal was observed.

Furthermore, the combined detection of IE mRNA and in vivo incorporated BrdU proved possible. The results showed that part of BrdU positive cells showed RNA expression as determined by the size and intensity of the nuclear hybridization spot and/or a cytoplasmic signal. Fig. 9 shows a BrdU positive cell expressing IE mRNA as indicated by the nuclear track like signal.

**Localization of IE, HEF and GAPDH mRNA**

The localization of IE mRNA was essentially as described previously (Raap et al., 1991). Hybridization experiments with the full plasmid probe containing both introns and exons on noninduced rat 9G cells, which were denatured prior to hybridization, revealed a fluorescent spot in all cell nuclei representing the integrated HCMV DNA fragment. This was also observed for cells which were induced for IE transcription by cycloheximide treatment and denatured prior to hybridization. However, the nuclear signals in part of those cells were clearly more intense and showed an elongated or path-like distribution. Most of the cells showing a strong nuclear fluorescence signal also showed a hybridization signal throughout the cytoplasm. Omission of denaturation in those preparations resulted in the disappearance of the small nuclear HCMV DNA spots while the nuclear and cytoplasmic RNA signals remained visible. The number of nuclear signals showing a track were about half of the number observed for heat-denatured cells. Control experiments with RNase and DNase treatments revealed that most of the nuclear signals were derived from RNA. After a DNase treatment of induced cells, which were denatured prior to hybridization, part of the cells still showed a weak elongated signal. RNase treatment of induced but non-denatured cells resulted in a strong decrease of fluorescence intensity of the nuclear signals.

To investigate whether intron sequences could be detected in nuclei of induced cells, in situ hybridization experiments were performed with the intron-specific probe. In part of the cells a nuclear, elongated dot-like, fluorescent signal could be detected and in addition, some small fluorescent spots were often observed in the vicinity of the main nuclear signal (Fig. 10, see also Fig. 7). No intron-specific signals were observed in noninduced cells.

Hybridization experiments with HEF and GAPDH mRNA-specific probes revealed that both types of mRNA can be detected in all cell types used. HeLa cells showed the strongest hybridization signals for HEF mRNA while the rat 9G cells showed strongest hybridization signals for GAPDH mRNA. Detection of elongation factor mRNA in rat 9G cells was possible, because of the high degree of homology of HEF with rat EF. In all cell types, the detection of HEF mRNA resulted in more intense hybridization signals than GAPDH mRNA, but signal intensities were much lower compared to hybridization signals of IE mRNA and 28 S rRNA. Hybridization signals of GAPDH and HEF mRNA were visible as a large number of small spots which were distributed throughout the whole cytoplasm (Fig. 11A,B). Cells which were heat-denatured prior to in situ hybridization showed, in addition to a cytoplasmic signal usually two, small nuclear spots probably representing the encoding genes. Nuclear hybridization signals were not observed when denaturation of cells was omitted. Control hybridizations with the pSS probe on HeLa and 5637 cells revealed no signals (Fig. 11C).

**DISCUSSION**

This paper describes an optimization of in situ hybridization procedures for the detection of mRNA expression in
cultured cells. With the optimized procedures it proved possible to detect simultaneously different RNA sequences by using haptenized and/or fluorochromized probes and to visualize nuclear pre-mRNA.

The fixation of the biological material is considered to be one of the most important steps in the in situ hybridization procedure, the choice of which is largely determined by the nature of the tissue or cells and by the procedure by which it is processed. This is also reflected in the numerous articles describing different fixation protocols. In general, however, there appears to be a consensus of opinion about optimal fixation procedures. Formaldehyde is most frequently used for tissue and cell fixation as it was shown to retain RNA within cells and to preserve cell morphology (Lawrence and Singer, 1985). However, when cells were fixed in formaldehyde we often obtained weak hybridization signals in spite of detergent treatments and, moreover, the results varied among cell lines. The commercial source of formaldehyde or the use of freshly prepared solutions from paraformaldehyde had only a minor influence on the hybridization results (see also Farquharson et al., 1990). Other studies showed that good hybridization results are obtained when cells are fixed in a protein precipitating fixative like ethanol/acetic acid instead of a crosslinking fixative (John et al., 1977; Kanz et al., 1988). We therefore evaluated a number of different fixatives and it appeared that the hybridization results improved considerably when acetic acid was added to the formaldehyde fixative.

A number of studies, including our own, have shown the
interdependence of fixation and permeabilization (Dirks et al., 1989; Larsson and Hougaard, 1990; Lawrence and Singer, 1985). We consistently found a considerable improvement of hybridization signals when formaldehyde-fixed cells were treated with pepsin and heat, prior to hybridization. Alternatively, detergents, proteinase K or freeze-thawing might be used as well to improve hybridization signals as shown in this and previous studies (Dirks et al., 1989; Larsson and Hougaard, 1990). Our experience is, however, that pepsin provides the best results after a careful titration of the concentration.

A comparison of different probe types revealed that synthetic oligonucleotides were less sensitive compared to plasmid, PCR and RNA probes, which might be due to the fact that fewer hapten or fluorochrome molecules are incorporated per molecule. However, the hybridization signals obtained with the 28 S rRNA oligonucleotide were visually of the same intensity as those obtained with the 28 S plasmid probe. Obviously, because of their optimal penetration capacity, oligonucleotide probes are suitable for the detection of relatively abundant RNA sequences (Dirks et al., 1990). Most strong hybridization signals were obtained with RNA probes, probably because the single-stranded probe sequences do not reassociate in the hybridization solution (Cox et al., 1984). A careful titration of probe concentration was, however, important, to avoid aspecific hybridization. With biotin- and digoxigenin-labeled probes similar hybridization results were obtained although biotin-labeled probes gave higher background. More serious problems concerning the specificity of biotin detection systems on tissue sections have been reported (Kelly et al., 1991). Hybridization signals were most intense when the biotin- or digoxigenin-labeled probes were detected by a three-step immunocytochemical detection system. Although the signal intensities of peroxidase and alkaline phosphatase reaction products as well as fluorescence are difficult to compare, we favour the use of fluorescence. Fluorescence microscopy affords a high resolution and different fluorochromes can be easily distinguished in multiple hybridization experiments.

We showed that both direct and indirect hybridization methods can be successfully used for the fluorescent detection of RNA sequences. The advantage of the use of directly labeled probes resides in the absence of any immunocytochemical background staining and the considerable time reduction of the procedure. The signal intensities obtained with rhodamine-labeled probes for IE mRNA or 28 S rRNA were almost as strong as those obtained by indirect techniques. These results indicate that the detection of hybrids by antibodies is not highly efficient, probably due to their limited accessibility, as we have observed before (Dirks et al., 1990).

In the past few years, a number of different approaches have been described that allow the simultaneous detection of different DNA or RNA sequences (Dirks et al., 1990, 1991; Haase et al., 1985; Hartford Svoboda, 1991; Hopman et al., 1986; Kiyama and Emson, 1990; Nederlof et al., 1989, 1990; Normand and Bloch, 1991; Ozden et al., 1990; Singer et al., 1987; Young, 1989). By combining biotin- and digoxigenin-labeled probes we were able to visualize intron- and exon-specific sequences simultaneously in one cell (Raap et al., 1991). Moreover, fluorochromized probes can be used in combination with haptenized probes or with other fluorochromized probes, in which case the best results were obtained by combining fluorescein- and rhodamine-labeled probes in the hybridization mixture. The use of coumarin-labeled probes should be restricted to the detection of the most abundant RNA sequences because coumarin has relatively low fluorescence and it is fast fading.

In addition to the simultaneous visualization of different RNA sequences it also proved possible to combine the tech-
nique of in situ hybridization with immunocytochemical techniques in order to detect simultaneously transcription and translation products. Moreover, it proved possible to combine the in situ hybridization technique with the visualization of in vivo incorporated BrdU. This combined detection offers the possibility of studying gene expression in relation to the stages of the cell cycle. The different nuclear staining patterns of BrdU can be used for the identification of specific periods within the S-phase (Fox et al., 1991; Van Dierendonck et al., 1989). The feasibility of combined DNA in situ hybridization and BrdU detection has been demonstrated before in order to correlate the growth rate of subsets of tumour cells, which were identified by chromosomal constitution (Van Dekken et al., 1991), and to study the dynamic organization of DNA replication in mammalian cell nuclei (O’Keefe et al., 1992).

By use of the optimized in situ hybridization procedure it proved possible to study the localization of IE mRNA in transfected rat 9G cells (see also Raap et al., 1991). It was observed that approximately 30% of the rat 9G cells expressed IE mRNA after induction of the cells by cycloheximide. Part of the cells, some of which also showed a cytoplasmic signal, showed a nuclear signal which was RNase sensitive, suggesting that nuclear RNA transcripts were visualized. Supportive evidence was obtained by double-hybridization experiments which showed that intron sequences were present in the nuclear spots. Occasionally, nuclear signals were observed which clearly showed a pathway or “track”. Our initial suggestion that these nuclear signals represent a transport route of mRNA is not unambiguously proven. The observations that undenatured cells still showed some “track”-like structures when they were treated with RNase and that the number of cells showing a track increased when the cells were denatured prior to hybridization leaves the possibility that DNA is visualized in an extended form. The increased number of elongated or track-like signals as a result of denaturation could be an effect of nuclear disruption by heating, which might be enhanced by cycloheximide treatment. The RNase sensitivity of most of the nuclear signals showed, however, that nuclear RNA is detected, possibly together with the encoding DNA. The observed nuclear signals in undenatured cells treated with RNase might indicate that part of the DNA was in a single-stranded form as a result of the fixation or hybridization procedure and therefore available for hybridization. On the other hand, it is still possible that the degradation of RNA by RNase treatment is not complete and that the increased number of track-like structures, which we observed after denaturation of the cells, is the result of a better accessibility of nuclear RNA for probe sequences. Our observation of track-like sequences is consistent with the observation by Lawrence et al. (1989) that Epstein-Barr virus (EBV) transcripts can be detected as a track in nuclei. More recently, a path-like distribution of nuclear transcripts of c-fos RNA has been observed in NIH-3T3 cells (Huang and Spector, 1991). Both studies suggest that the track-like signals represent the transport of nascent transcripts to the cytoplasm. Our results obtained with intron- and exon-specific probes in a double-hybridization experiment suggest that pre-mRNA is present in the entire length of the track, as far as the nuclear envelope. Because no gradual decrease in the intron-specific hybridization signal was observed towards the nuclear envelope, we cannot draw the conclusion that the tracks observed for IE mRNA represent a transport route during which splicing takes place. It is still possible that we observed nascent transcripts along extended IE DNA on which splicing occurs. This would be consistent with a number of studies which showed that splicing factors are associated with nascent transcripts (Fakan et al., 1986; Osheim and Beyer, 1991).

The small fluorescent dots which were occasionally found surrounding the tracks might represent intron sequences which are spliced out. It is conceivable that spliced intron sequences diffuse throughout the nucleus after being released from the spliceosome. More specific distribution patterns of introns have been observed recently. The first intron of the AchR α subunit RNA was found localized to the nuclear membrane of multinucleated myotubes (Berman et al., 1990) and introns excised from the Delta primary transcript were found localized to two foci, probably near sites of Delta transcription, in nuclei of Drosophila embryos (Kopczynski and Muskavitch, 1992). That spliced introns can be relatively stable and accumulate in nuclei has recently been shown for a T cell receptor-β intron in mammalian T cells (Qian et al., 1992). In order to investigate the spatial organization of IE DNA in relation to its transcribed RNA, double-hybridization experiments should be performed with DNA- and RNA-specific probes. Furthermore, probes are currently designed which allow us to discriminate between pre- and mature mRNA in order to get more information about the actual site where splicing takes place (F. M. van de Rijke, unpublished observation).

In situ hybridization with intron-specific probes proved to be not only a useful tool in mRNA splicing studies but also very useful for the detection of transcription regulation because it allows the visualization of newly transcribed RNA sequences before they are processed and transported to the cytoplasm (Fremeau et al., 1986; Herman et al., 1991).

Obviously, the study of molecular events at the cellular level requires a high sensitivity of the in situ hybridization technique. The ultimate sensitivity of the technique is not determined but, as illustrated in this study, the housekeeping gene HEF and GAPDH mRNAs can be easily detected. For comparison, HEF mRNA can be detected with a 32P-labeled probe on a Northern blot of 10 µg total RNA after 5 h of exposure to an X-ray film.

In conclusion, the in situ hybridization method as described in this study allows the detection of abundant cytoplasmic and nuclear RNA sequences with high signal-to-noise ratios.

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