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Nuclear Matrix Bound Fibroblast Growth Factor Receptor Is Associated With Splicing Factor Rich and Transcriptionally Active Nuclear Speckles

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Abstract We have used confocal microscopy combined with computer image analysis to evaluate the functional significance of a constitutively expressed form of the receptor tyrosine kinase FGFR1 (fibroblast growth factor receptor 1) in the nucleus of rapidly proliferating serum stimulated TE 671 cells, a medullobastoma human cell line. Our results demonstrate a limited number of large sites and numerous smaller sites of FGFR1 in the nuclear interior. The larger sites showed virtually complete colocalization (>90%) with splicing factor rich nuclear speckles while the smaller sites showed very limited overlap (<20%). Similar results were found for several other proliferating cell lines grown in culture. An in situ transcription assay was used to determine colocalization with transcription sites by incorporating 5-bromouridine triphosphate (BrUTP) followed by dual staining for BrUTP and FGFR1. These results combined with those from using an antibody against the large subunit of RNA polymerase II suggest a significant degree of colocalization (26–38%) over both the large and small sites. No colocalization was detected with sites of DNA replication. The spatial arrangements of FGFR1 sites and colocalization with nuclear speckles were maintained following extraction for nuclear matrix. Moreover, immunoblots indicated a significant enrichment of FGFR1 in the nuclear matrix fraction. Our findings suggest an involvement of a nuclear matrix bound FGFR1 in transcriptional and RNA processing events in the cell nucleus. We further propose that nuclear speckles, aside from a role in transcriptional/RNA processing events, may serve as fundamental *regulatory factories* for the integration of diverse signaling and regulatory factors that impact transcription and cellular regulation. J. Cell. Biochem. 90: 856–869, 2003.

Key words: FGFR1; cell nucleus; nuclear matrix; nuclear speckles; splicing factors; transcription sites; RNA polymerase II; laser scanning confocal microscopy; microscopy image analysis

Fibroblast growth factors (FGFs) comprise a large family of developmental and physiological signaling molecules involved in embryogenesis, wound repair, angiogenesis, tumor growth, and other biological and pathological processes related to cell growth and proliferation [Burgess, 1989]. FGFs mediate their function by binding to a class of high affinity membrane spanning glycoproteins termed fibroblast growth factor receptors (FGFRs) and low affinity heparin sulfate proteoglycans present on the cell surface [Galzie et al., 1997; Ornitz, 2000]. Four different FGFRs (1–4) have been cloned that share homology in both structure and amino acid sequence. The general structure consists of an extracellular region, composed of three immunoglobulin (Ig)-like domains, a transmembrane region, and an intracellular tyrosine kinase

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domain [Jaye, 1992; Johnson et al., 1993; Green et al., 1996].

Unmodified FGFR1 encodes an 822 amino acid protein with an approximate molecular weight of 100 kDa. However, post-transcriptional and post-translational modifications, such as RNA splicing and glycosylation, result in the detection of multiple forms of the receptor within a cell. In addition to this extracellular paracrine role, FGFR1 is believed to play a major role in an autocrine mechanism of regulating cell growth and division in which the translocation of FGFR1 to the cell nucleus is a critical step. Details of this autocrine model, termed "integrative nuclear FGFR1 signaling" (INFS), and the experimental basis supporting the model, can be found in an accompanying review article [see Stachowiak et al., 2003a]. In brief, translocation of FGFR1 into the cell nucleus following stimulation of target cells with intracellular FGFs or other regulatory factors activates changes in gene expression via multiple interactions with a discrete subset of signaling and transcriptional regulatory factors. These events, in turn, modulate the expression of other signaling and regulatory factors which together mediate the overall genomic reprogramming for the process(es) being activated [e.g, cell growth, proliferation or the synthesis and secretion of specific signaling molecules; Stachowiak et al., 2003a].

In support of this model is a growing body of evidence in a variety of cellular and tissue systems which demonstrates a correlation between the presence of FGFR1 in the cell nucleus and cellular proliferation and the requirement of FGFR1 translocation into the nucleus for both cellular activation and the specific induction of certain genes [see Stachowiak et al., 2003a for review; Tessler and Neufeld, 1990; Rider et al., 1995; Maher, 1996a; Stachowiak et al., 1996a, 1997a; Thompson et al., 1998; Liu and Zhu, 1999; Peng et al., 2001, 2002; Reilly and Maher, 2001]. In addition, Stachowiak et al. [1997a] found that nuclear FGFR1 is constitutively expressed in rapidly proliferating glioma cells. Aside from further supporting a correlation between nuclear bound FGFR1 and cell proliferation, these findings suggested the possibility that the continuous growth and proliferation characteristic of many immortalized and neoplastically transformed cells in culture, may be due, in part at least, to the presence of active FGFR1 in the nucleus which contributes to keeping the genome programmed to a locked-in proliferative state.

With the above in mind, we have used a combination of laser scanning confocal microscopy and computer image analysis to investigate the distribution of FGFR1 relative to structural and functional components in the nucleus of rapidly proliferating medulloblastoma cells (TE 671) grown in culture. Our results demonstrate the presence of a constitutive form of FGFR1 in the nucleus of medulloblastoma cells, as well as in a variety of other proliferating cells grown in culture. This constitutively expressed nuclear FGFR1 is tightly bound to the nuclear matrix architecture and co-localizes with splicing factor-rich nuclear speckles and, to a more limited extent, with sites of mRNA transcription in the nucleus.

MATERIALS AND METHODS

Cell Culture and Fractionation

Human medulloblastoma cells (TE 671), mouse 3T3 fibroblasts, NHF1 normal diploid human fibroblasts and HeLa cells were grown as monolayers and maintained in a rapidly proliferating state in DMEM (GIBCO, Grand Island, NY) supplemented with 10% FBS (GIBCO) and non-essential amino acids (GIBCO). Human astrocytes were obtained from Clonetics (San Diego, CA) and were cultured in Wa87/3 medium supplemented with 20% FBS [Stachowiak et al., 1997a; Moffett et al., 1998] For preparing cell extracts, cells were trypsinized and spun down at 1,000*g* for 5 min. The cells were lysed by incubation with lysis buffer for 5 min on ice (10 mM Tris, pH 8.5, 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP-40). The cell lysate was centrifuged at 5,000*g* for 5 min to pellet nuclei. Nuclear matrix preparation was performed according to the procedure of Belgrader et al. [1991]. Briefly, nuclei were isolated by disruption and subsequent passages of TE cells through a 22-gauge syringe. Isolated nuclei were treated with DNase I (Sigma, 30 U/mg DNA) on ice for 30 min and extracted with 0.6 M ammonium sulfate in 0.2 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4. The final nuclear matrix pellet was suspended in 0.2 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4 containing 50% glycerol. Cell extracts from human endothelial kidney cells (HEK 293 cells) transfected with a plasmid encoding the full-length FGFR1 were obtained from Sigma. Protein determinations

were performed with the BCA kit (Pierce, Chester, UK).

For in situ nuclear matrix preparations, cells growing on coverslips were permeabilized in glycerol buffer (20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 25% glycerol, 0.5 mM EGTA, 0.5 mM PMSF) containing 0.5% Triton X-100. DNA was digested with DNase I (Sigma, 25 U/ml) for 30 min in glycerol buffer (minus EGTA) on ice followed by extraction with either 0.2 or 0.6 M ammonium sulfate in glycerol buffer. The cells were then fixed with 4% paraformaldehyde and immunostained using polyclonal FGFR1 antibodies (Sigma).

Western Blotting

Denaturing SDS–polyacrylamide gel electrophoresis (PAGE) was performed using a minigel apparatus (BioRad, Hercules, CA). Samples were run on a 7.5% acrylamide gel and transferred to PVDF membrane. The membrane was blocked using a mixture of 3% BSA and 1% nonfat dry milk in TBS-buffer. Following primary and secondary antibody incubation the chemiluminescent signals were detected using the ECL kit (Amersham, Buckinghamshire, UK).

Labeling for Immunofluorescence Microscopy

Cells were typically grown on coverslips for 48 h, fixed in 4% paraformaldehyde for 10 min at room temperature and permeabilized with 0.5% Triton X-100 in PBS buffer for 5 min. Primary and secondary antibodies were diluted in PBS and incubated at room temperature for 1 h. When performing double immunofluorescent assays we used a sequential method of staining that alternatively used primary antibody and secondary antibody combinations. Nascent RNA transcription was detected using an in situ procedure [Wei et al., 1998, 1999] following incorporation of BrUTP (Sigma). Briefly, permeabilized cells were incubated with transcription buffer containing BrUTP in addition to the other three NTP's for 30 min at room temperature [Wei et al., 1998, 1999]. Following RNA synthesis, the cells were fixed and stained with antibodies against BrUTP (SeraLab) and FGFR1 (Sigma). DNA replication sites were labeled following a 10 min pulse with BrdU as described by Ma et al. [1998]. FGFR1 antibody obtained from Sigma recognized a 14 amino acid sequence (360–373) on the N-terminal extracellular domain of the receptor. We obtained similar results as shown in this manuscript with a FGFR1 antibody generated against the C-terminal region of FGFR1 [Hanneken et al., 1995]. Antibodies to splicing factors (SC-35 and Y12) were obtained from Dr. T. Maniatis and Dr. J. Steitz, respectively. SRm 160 was kindly provided by Dr. B. Blencowe. The Br-UTP antibody was obtained from SeraLab. The following secondary antibodies were used in all experiments: anti-rabbit biotin (Jackson Immunoresearch), Strepavidin-Texas red (GIBCO), anti-mouse FITC (Jackson Immunoresearch), anti-rabbit HRP (Sigma).

Microscopy and Image Analysis

Confocal images were collected using a Bio-Rad MRC-1024 three channel laser scanning confocal microscope imaging system equipped with a Nikon Optiphot 2 microscope, a Nikon 60×, 1.4 NA objective, and a krypton/argon laser ($l = 488/565$ nm). Optical sections of 512×512 were collected at 0.5 μm intervals. In some instances, optical sections were collected with a Zeiss Photomicroscope III Fluorescence Microscope equipped with a high resolution CCD camera (Princeton Instruments) and a z-axis controller. Segmentation of confocal images was performed using a threshold (IPLab, Scanalytics, Fairfax, VA) and an in-house developed, spot-based algorithm [Samarabandu et al., 1995]. A combination of the two methods allowed us to accurately segment the large and small intensity sites in the image. The area occupied by the segments was determined by counting the number of pixels enclosed within the individual segments.

RESULTS

Subcellular Localization of FGFR1

Whole cell extracts and purified nuclei were prepared from rapidly dividing TE 671 human medulloblastoma cells and immunoblotted (using equal amounts of protein) with an anti-receptor antibody (Sigma) raised against amino acids 360–373 of the extracellular region of human FGFR1. Two major bands were detected at approximately 110 and 67 kDa in whole cell extracts which were several fold enriched on a protein basis in the corresponding nuclear fractions, (Fig. 1, lanes 1 and 2). A faintly staining band migrating around 140 kDa was routinely detected in the nuclear fraction but was barely detectable in the whole cell extract.

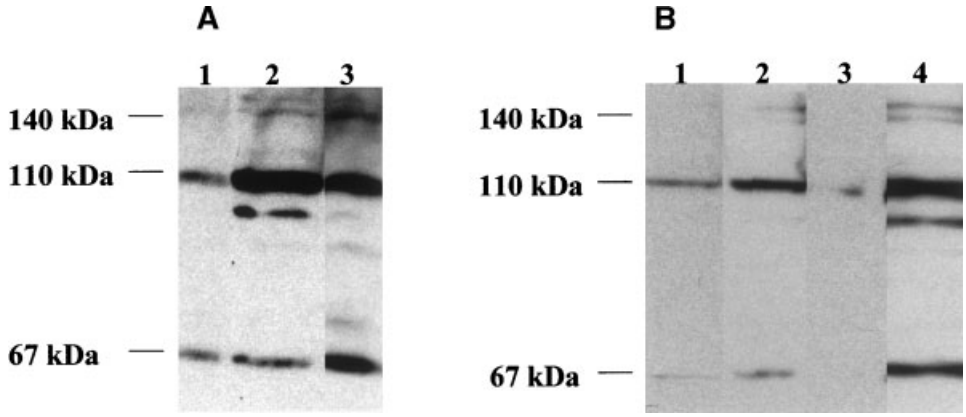

Fig. 1. Western blot analysis of FGFR1 in subcellular fractions from TE 671 cells. Protein (50 μg) from each fraction were subjected to SDS–PAGE, transferred to PVDF membrane and probed with a polyclonal antibody to FGFR1. **A**: **lane 1**, total cell extract; **lane 2**, purified nuclear fraction; **lane 3**, total cell extract from HEK 293 cells transfected with a full-length FGFR1 cDNA construct; **B**: **lane 1**, total cell extract; **lane 2**, purified nuclear fraction; **lane 3**, high salt extract; **lane 4**, final nuclear matrix. Molecular weight marker positions are shown on the left side of each immunoblot.

A signal at approximately 90 kDa was also often observed in the nuclear fraction but not in the whole cell extract. Similar results were found in fractions obtained from human glioma cell lines U251, U763, and U767 (results not shown). The 140, 110, and 67 kDa bands were also detected in a control lane (Fig 1, lane 4) using cell extracts from human endothelial kidney cells (HEK 293 cells) transfected with a plasmid encoding the full-length FGFR1.

Association of Nuclear FGFR1 With the Nuclear Matrix

Nuclear matrix was isolated from exponentially dividing TE 671 cells using an established protocol that minimized protein degradation during the isolation procedure (see "Materials and Methods"). Typically 15–20% of the total nuclear protein was recovered in the isolated nuclear matrix fraction. Equal protein amounts of the different fractions from the extraction protocol were run on a SDS–PAGE gel and probed with the anti-FGFR1 antibody. The characteristic bands of 110 and 67 kDa were detected with a several fold enrichment in the nuclear matrix compared to the total nuclear fraction (Fig. 1B, lanes 2 and 4). A doublet of high molecular weight bands was also detected between 140–170 kDa and enriched in the nuclear matrix. The 90 kDa band was barely detectable in this total nuclear fraction but was a prominent band in the nuclear matrix. Only a small quantity of FGFR1 was observed in the high salt washes at the 110 kDa position (Fig. 1B, lane 3) indicating that the receptor in the nucleus remained tightly bound to the nuclear matrix following the extraction protocol.

Immunolocalization of FGFR1

After fixation with paraformaldehyde, FGFR1 sites were immunolocalized in medulloblastoma TE 671 cells using the anti-FGFR1 polyclonal antibody (see "Materials and Methods"). Optical sections were collected and the images analyzed as described in "Materials and Methods." Using this approach, we found strong FGFR1 staining that was localized to the nucleus in medulloblastoma cells. Greater than 90% of the cells had large discrete punctate staining sites that were distributed throughout the nucleus and excluded from the nucleolus (Fig. 2A). Approximately 20 large discrete FGFR1 sites were counted in a typical midplane optical section through the cell nucleus. In addition, a much larger number of smaller FGFR1 stained sites (>100) were detected throughout the nucleus. The relative intensity of staining in the large granular sites was roughly fivefold greater than that measured in the rest of the nucleus.

A similar distribution of FGFR1 sites was observed in the nuclei of Hela cells (Fig. 2B), activated human astrocytes (Fig. 2C), mouse 3T3 cells (Fig. 2D), and normal diploid human fibroblasts (Fig. 2E). Although there were small differences in the size and number of FGFR1 sites among different cell lines, we failed to observe any significant change in the distribution of sites, which remained predominantly

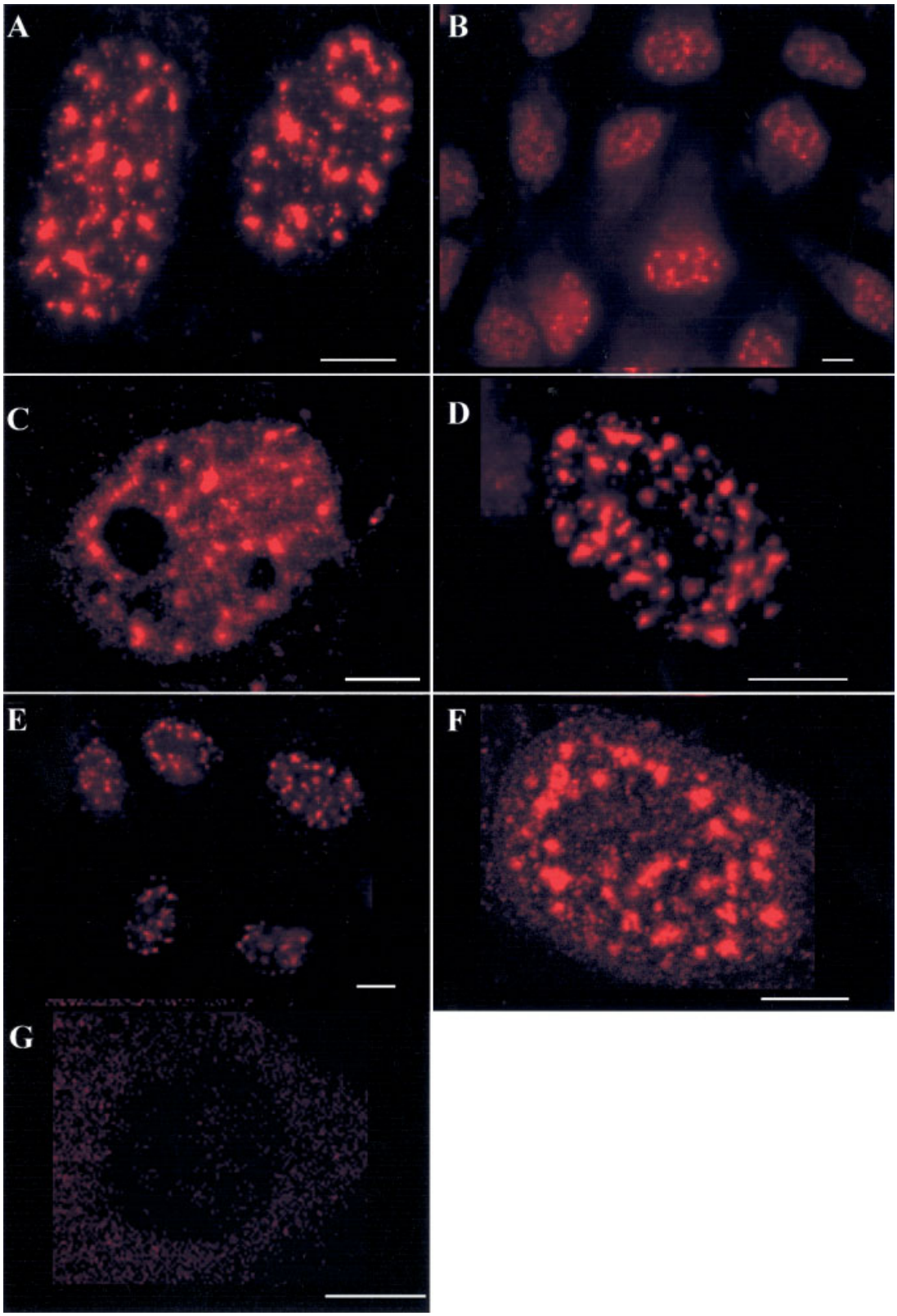

Fig. 2. Immunolocalization of FGFR1. Exponentially dividing cells on coverslips were fixed, permeabilized and labeled with anti-FGFR1 antibody. Single mid-plane optical sections using confocal laser scanning microscopy were obtained from TE 671 (**A**) and NHF1 cells (**D**). A fluorescence microscope mounted with a CCD camera was used to obtain the images from Hela cells (**B**), astrocytes (**C**), and NIH3 T3 cells (**E**). **F**: This is a confocal section of a TE 671 cell following extraction on the cover slip for nuclear matrix. **G**: This is a confocal section through a TE 671 cell in which an excess of synthetic peptide to FGFR1 is added prior to labeling with anti-FGFR1. Note that the staining in the nuclear area is below the background staining in the cytoplasm. This image was overexposed compared to the other images in order to visualize the cytoplasmic background staining. Bars, 5 μm.

granular and extranucleolar. Occasionally we noticed that the larger domains of FGFR1 staining appeared to consist of several small subdomains. Figure 2D contains an insert showing a single granular domain composed of several subdomains.

Since our immunoblot experiments suggested that FGFR1 was predominately associated with the nuclear matrix, we examined the immunolocalization of FGFR1 following extraction of TE 671 cells grown on coverslips for a standard in situ nuclear matrix preparation using DNase I digestion and 0.6 M ammonium sulfate treatment (see "Materials and Methods"). The staining pattern was strikingly similar to that observed in whole cells (Fig. 2F) and over 90% of the observed nuclear matrices contained this characteristic pattern. Identical staining patterns were obtained (not shown) when a lower salt concentration was used for extraction (0.2 M ammonium sulfate) or when the DNase I digestion step was omitted to prepare "DNA-rich" nuclear matrices (Berezney and Buchholtz, 1981). The FGFR1 staining patterns could be abrogated by use of a competitive inhibitor. Under these conditions, even upon extended exposure, only background staining was detected in the cytoplasm with virtually no signal detected in the cell nucleus (Fig. 2G).

Colocalization of FGFR1 to Sites of Nuclear Speckles

In separate assays, monoclonal antibodies to splicing factors SC-35, SRm 160 and the Sm class of snRNP proteins (Y12) were used in conjunction with anti-FGFR1 antibody to double immunolabel medulloblastoma TE 671 cells. Following double labeling, confocal image sets were obtained by optical sectioning of the nucleus and the individual channels merged to determine colocalization.

Figure 3 shows representative individual two channel and merged images from colocalization experiments using SRm 160 (panel A), Y12 (panel B), and SC-35 (panel C) antibodies. All three splicing factor antibodies showed characteristic strong staining of speckled sites (white arrow) and a weak diffuse staining of the nucleoplasm (magenta arrow, Fig. 3A). A merge of the two channels indicated that the speckled sites of staining in the nucleus were also highly enriched in FGFR1. We noticed that the intensity of staining between the individual channels varied resulting in different shades of yellow in the colocalized regions. In addition, a visual inspection of the merged images in Figure 3 showed that the spatial organization of speckles and FGFR1 sites, although highly colocalized, was not identical. In many cases, we found that large granular sites of FGFR1 staining extended slightly beyond the nuclear speckles stained with splicing factor antibodies or vice-versa. In addition, a more limited association was seen between the two channels in the regions of the nucleus outside of the speckled domains. Virtually identical staining patterns were found when cells were extracted for nuclear matrix and then dual labeled for FGFR1 and SC-35 (Fig. 3E).

Three-Color Dual Segmentation and Quantitative Image Analysis of FGFR1 and Splicing Factor Rich Sites

The preceding immunolocalization studies (Fig. 3A–C) prompted us to perform three-color segmentation and image analysis in order to estimate the extent of colocalization between FGFR1 sites and splicing factor-rich nuclear speckles of the nucleus. Two different segmentation methods were applied that allowed us to differentiate the small and large staining sites based on their relative sizes (see "Materials and Methods"). Using this approach we found that the average area occupied by the individual large granular sites was typically 20–30 fold greater than that of the individual smaller sites. As a total population, the limited number of large FGFR1 labeled sites occupied 4–5 fold more area in the nucleus than the much larger number of smaller FGFR1 stained sites. Following segmentation, the contours representing the two color channels were merged and the extent of overlap determined based on the area (in pixels) that was enclosed within the individual segments.

Figure 3C,D show individual two channel images of the original optical section in which FGFR1 was dual labeled with the splicing factor SC-35 (C panel) and the results following dual segmentation (D panel). The area occupied by the segments was determined based on the number of enclosed pixels. Identical analysis was applied to images labeled following extraction for nuclear matrix Results from 10–12 images for each of the three splicing factors for total TE 671 cells and nuclear matrix, respectively, were averaged and plotted in Figure 4. In whole cells over 90% of the area

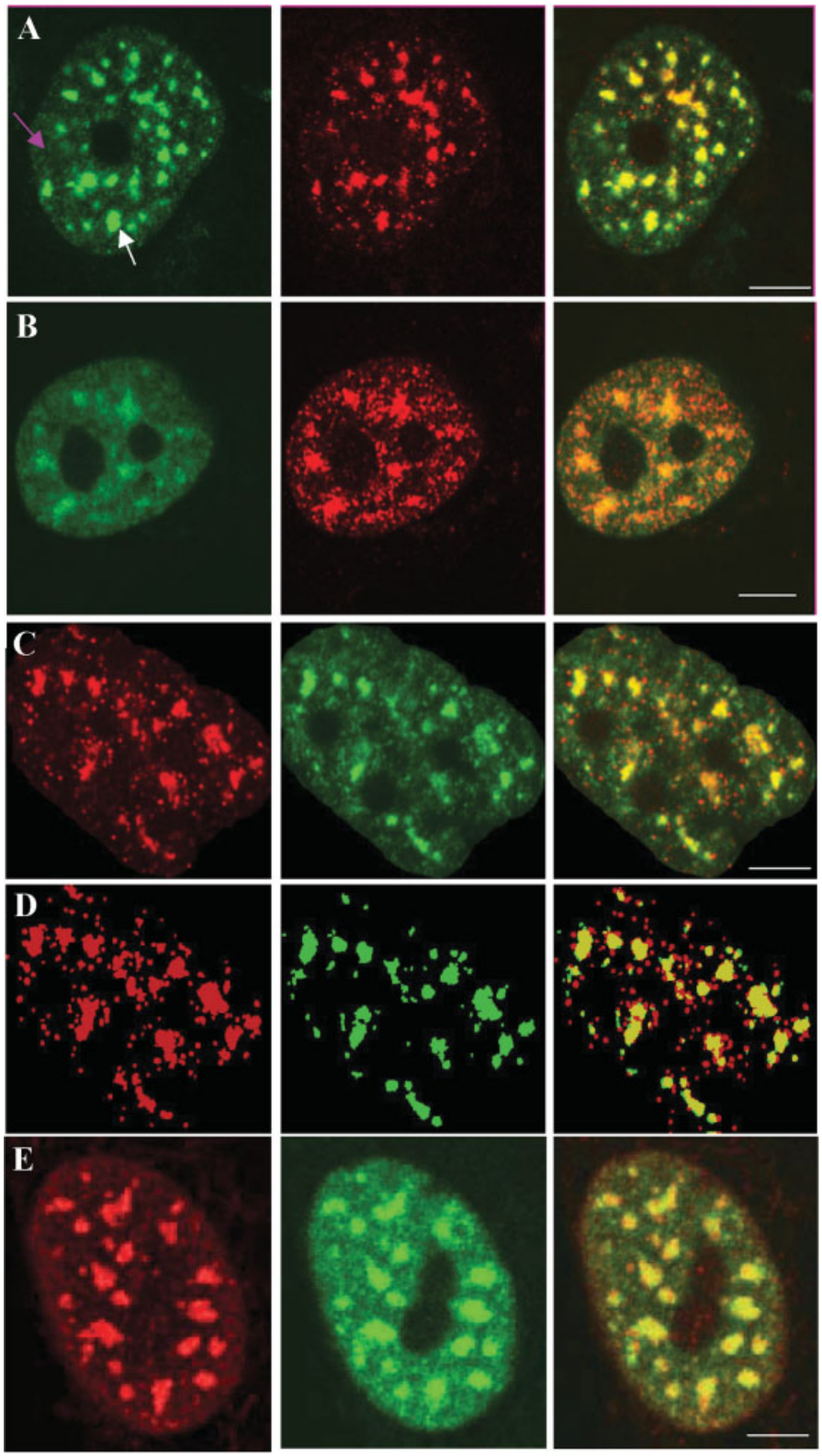

Fig. 3. Colocalization of FGFR1 with splicing factors. TE 671 cells growing on coverslips were fixed, permeabilized and double labeled sequentially using antibodies to splicing factors and FGFR1. The left (splicing factors) and middle lanes (FGFR1) are single mid-plane optical sections obtained from the dual color channels. The digital merges of the dual channel images are shown in the right lanes. Three splicing factor antibodies, SRm-160 (**Panel A**), Y12 (**Panel B**), and SC-35 (**Panel C**), were used in these studies. **Panel D** shows the segmented images corresponding to panel C. **Panel E** shows FGFR1 and SC-35 staining following extraction of TE 671 cells for nuclear matrix. Bars, 5 μm.

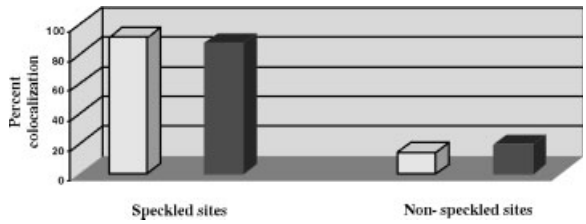

Fig. 4. Quantifying the colocalization of FGFR1 with splicing factors. Three color dual segmentation was performed on the confocal images to determine the degree of colocalization between FGFR1 and the splicing factors used in Figure 3. Representative results for this segmentation is shown in Figure 3, panel D for the SC-35 antibody. Following segmentation, the total area occupied by small or large FGFR1 sites was determined based on the number of pixels enclosed within their respective contours. The percent of this area, occupied by large or small FGFR1 sites that colocalized with splicing factors SRm 160, SC-35, and Y12 was then plotted as a histogram. The y-axis represents the percent of colocalization as a measure of the total area enclosed within the large or small contours. The light and dark bars correspond to whole cells and nuclear matrix, respectively and represent the average of 10–12 separate determinations for each of the three splicing factors.

occupied by the large granular FGFR1 sites colocalized with sites of splicing factor rich speckles (Fig. 4). Less than 20% of the total area occupied by the smaller FGFR1 sites colocalized with splicing factors found outside of the nuclear speckles (Fig. 4). The findings for nuclear matrix were similar to unextracted cells (Fig. 4).

Association of FGFR1 With Sites of Transcription

TE 671 cells growing on coverslips were permeabilized and labeled with 5-bromouridine triphosphate (BrUTP) using a standard in situ run-on transcription assay followed by staining for FGFR1 (see "Materials and Methods"). Sites of BrUTP incorporation, representing nascent transcripts [Wansink et al., 1993; Wei et al., 1998], and FGFR1 were detected using monoclonal and polyclonal antibodies, respectively. Optical sections were then collected and analyzed for colocalization between the individual channels.

Nascent RNA transcripts labeled by BrUTP incorporation showed a typical punctate staining pattern consisting of numerous foci scattered throughout the nucleus (Fig. 5A). Segmentation and 3-D reconstruction of the optical sections revealed an average of approximately 2,000 transcription sites in the interphase nucleus of proliferating TE 671 cells (results not shown) which is similar to what was previously reported in mouse 3T3 cells using the same segmentation and 3-D reconstruction algorithms [Wei et al., 1999]. Strong nucleolar staining was also observed indicating run-on transcription from rRNA genes [Wei et al., 1998, 1999]. Merging of the two channel images showed limited colocalization between sites of transcription and FGFR1 (Fig. 5C). In particular, significant colocalization is observed between transcription sites and FGFR1 along the periphery (Fig. 5D and E, arrowheads) as well as in the interior (Fig. 5D and E, arrows) of the nuclear speckles. The interior localization of numerous transcription sites was confirmed by tracking the sites in consecutive optical sections through individual FGFR1 stained nuclear speckles as previously reported [Wei et al., 1999]. The majority of transcription sites detected inside the speckled regions also extended to the periphery of the speckle (arrow in Figure 5E). Less colocalization of transcription with FGFR1 sites was observed outside of the speckled regions. Closer inspection revealed that the smaller FGFR1 sites outside of the speckles were typically in juxtaposition with transcription sites. This is illustrated in Figure 5F where a single FGFR1 site (green) is surrounded by three transcription sites (red). Colocalization was quantified by our dual segmentation procedure (see Materials and Methods) and showed an average of 38% and 26% colocalization of FGFR1 with transcription sites in the speckled and nonspeckled regions, respectively (Fig. 6)

We next examined the spatial relationship of RNA polymerase II (pol II) with FGFR1 using an antibody specific to the hyperphosphorylated C-terminal domain of the pol II large subunit [Mortillaro et al., 1996; Patturajan et al., 1998]. This is the form of RNA polymerase II that is active in transcriptional elongation. As expected, the pol II sites are devoid in the nucleolar regions (Fig. 5G). Co-localization of FGFR1 with pol II sites is similar to that seen for transcription sites with a particularly significant association of pol II within and along the periphery of FGFR1 decorated nuclear speckles (Fig. 5I,J). Quantification with our segmentation programs revealed that approximately 33% and 30% of the FGFR1 colocalized with pol II sites in speckled and nonspeckled regions, respectively (Fig. 6). Similar levels of colocalization were determined using antibodies against different regions of FGFR1 (results not shown).

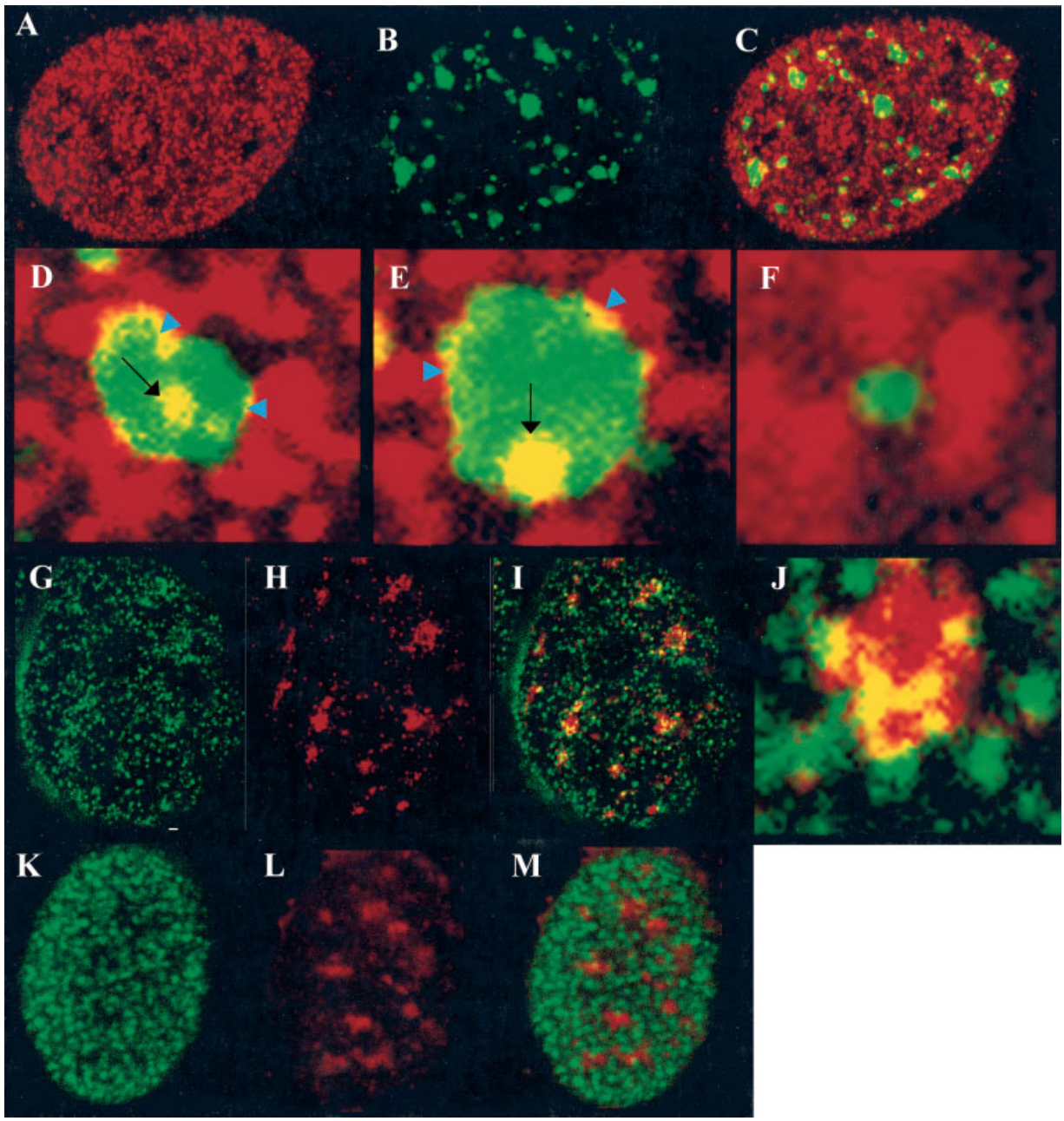

Fig. 5. Colocalization of FGFR1 with sites of transcription, RNA polymerase II and DNA replication. Following nascent RNA synthesis, TE 671 cells were fixed and labeled with antibodies to detect sites of transcription and FGFR1. Optical sections of cell double labeled for transcription (**A**), and FGFR1 (**B**) were digitally merged in (**C**). Regions of the overlaid image in (C) were enlarged (**D**, **E**) to the show the presence of transcription sites (red) within (arrows) and on the periphery (arrowheads) of the FGFR1-labeled nuclear speckles (green). **Panel F** shows a small FGFR1 site (green) in close proximity but not overlapping with several transcription sites (red). TE 671 cells were double labeled with antibodies against RNA polymerase II (green) (**G**), FGFR1 (red) (**H**), and the images merged in (**I**). An enlarged region of the nucleus shows the colocalization over a large FGFR1 speckle site (red) and sites of RNA polymerase II (green) (**J**). Extensive colocalization along the periphery and within the interior of the speckle is indicated by the yellow color. The bottom panel shows confocal sections of cells labeled for DNA replication sites by pulsing with BrdU (green) (**K**) and FGFR1 (red) (**O**). A digital merge of the two channels is shown in (**M**).

The potential functional significance of the association of FGFR1 with active sites of transcription is further supported by our results on dual labeling of replication sites and FGFR1 in TE 671 cells. Despite the presence of approximately 1,000 sites of replication (determined by 3-D analysis of segmented images), no colocalization was detected by visual inspection of the

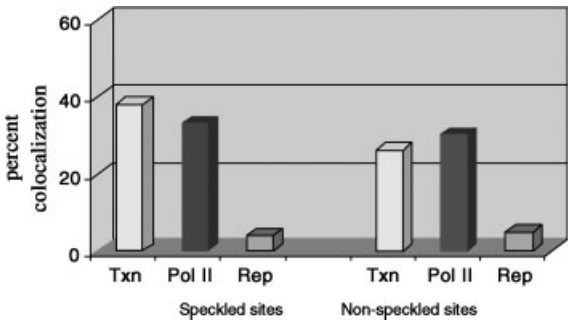

Fig. 6. Colocalization of large and small FGFR1 sites with sites of transcription, RNA pol II and DNA replication. Using three-color dual segmentation, the areas occupied by FGFR1 and transcription sites, replication sites and RNA pol II were determined based on the number of pixels enclosed within the contours. The extent of colocalization among large or small FGFR1 sites with sites of transcription, replication and pol II were measured separately and the results plotted as a histogram. The y-axis represents the percent of colocalization as a measure of the total area enclosed within the large (speckled sites) or small (nonspeckled sites) contours.

images (Fig. 5K–M). Consistent with direct inspection, quantification of overlap following segmentation resulted in very low values (<5%) which were below the confidence level for significant colocalization (Fig. 6).

DISCUSSION

We have examined in detail the localization of FGFR1 in the rapidly proliferating human medulloblastoma cell line TE 671. Laser scanning confocal microscopy demonstrated a limited number (approximately 20 in a typical midplane optical section) of large granular sites of FGFR1 staining that were present throughout the nucleoplasm and excluded from the nucleolus. In addition to these large granular sites, a greater number (<100) of smaller stained foci was seen throughout the extranucleolar region. Examination of several rapidly proliferating normal and transformed cell lines, ranging from mouse to human cells showed a similar pattern of staining. The presence of a similar nuclear staining pattern in the great majority of examined cells (>90%), leads us to conclude that nuclear bound FGFR1 is a constitutive property of rapidly proliferating cells. Consistent with these findings, a constitutively expressed FGFR1 was previously reported by Stachowiak et al. [1997a] in rapidly proliferating glioma cells.

While FGFR1 staining was not detected on the cell surface of any of the cells investigated, our results do not preclude the existence of FGFR1 on the cell surface. Rather they indicate, that in rapidly proliferating cells, the level of FGFR1 on the cell surface is much lower compared to the discrete and heavily stained sites detected in the cell nucleus. Thus we favor the interpretation that the immunofluorescence labeling protocol that we used in this study (standard secondary antibody procedure, see Materials and Methods) is not sensitive enough to enable detection of the cell surface population of FGFR1.

Immunoblot analysis revealed that the major band for FGFR1 migrated at approximately 110 kDa in the total TE 671 cell extract which was enriched several fold on a protein basis in the corresponding nuclear fraction. Less intense staining bands were also detected at approximately 170, 140, 90, and 67 kDa. Our results are consistent with a number of studies demonstrating multiple forms of FGFR1 that vary from approximately 170–90 kDa depending on the tissue or cell source and, possibility, the SDS–PAGE system [Rider et al., 1995; Maher, 1996a; Stachowiak et al., 1996a,b, 1997a; Reilly and Maher, 2001; Peng et al., 2001, 2002]. The higher molecular weight components (>100 kDa) are likely a result of different degrees of glycosylation of the protein [Feige and Baird, 1988; Prudovsky et al., 1996; Stachowiak et al., 1997b] and/or alterative splice variants [Johnson and Williams, 1993; Reilley and Maher, 2001].

The significance of the 67-kDa band in our preparations is less clear. Several studies have detected lower molecular weight forms of FGFR1 (~45–90 kDa) that are presumed to arise by either alternative splicing or activation of secondary translational initiation sites within the coding sequence [Jaye et al., 1992; Pasumarthi et al., 1995; Rider et al., 1995; Green et al., 1996; Galzie et al., 1997]. While we can not rule out the possibility that the 67 kDa is a degradation product of the, for example, major 110 kDa protein band, it was consistently detected in numerous extract preparations and was significantly enriched in the corresponding nuclear fractions. In contrast, the approximately 90-kDa band was sometimes not detected and was more variable in staining intensity among different nuclear preparations. This is consistent with the observation that the

approximately 90-kDa band represents the nonglycosylated receptor [Stachowiak et al., 1997b]. Moreover, the 170, 140, 110, 90, and 67 kDa bands were detected in an extract derived from cells transfected with the cDNA encoding the full length FGFR1, albeit the 90-kDa band was considerably weaker in intensity.

Several nuclear functions such as DNA replication, transcription and RNA splicing, along with the factors that mediate them, are associated with the nuclear matrix- an underlying nuclear structure that is observed following appropriate extraction of isolated nuclei [Berezney and Coffey, 1975, 1977; Smith and Berezney, 1982; Spector et al., 1983; Tubo and Berezney, 1987a,b; Nakayasu and Berezney, 1989; Xing and Lawrence, 1991; Blencowe et al., 1994; Mortillaro et al., 1996; Nickerson et al., 1997; Patturajan et al., 1998; Wei et al., 1999; for reviews see Berezney et al., 1995; Berezney and Wei, 1998; Cook, 1999; Stein et al., 1999, 2000; Berezney, 2002]. This prompted us to investigate the association of FGFR1 with the nuclear matrix. Biochemical fractionation of the isolated TE 671 nuclei combined with immunoblotting indicate a several fold enrichment of the multiple FGFR1 staining bands at approximately 170, 140, 110, 90, and 67 kDa in the nuclear matrix fraction. These results are consistent with previous studies reporting the association of the FGFR1 with the nuclear matrix using different antibodies against N- and C-terminal regions of FGFR1 [Maher, 1996b; Stachowiak et al., 1996a,b]. Taken together, these previous findings along with those of this study, confirm that the full-length FGFR1 associates with the nuclear matrix.

Immunofluorescent analysis using laser scanning confocal microscopy demonstrated that the overall staining intensity and spatial arrangements of FGFR1 detected on the nuclear matrix was indistinguishable from that observed in intact cells. This is consistent with the immunoblot results indicating that the great majority of FGFR1 is maintained on the nuclear matrix following extraction and further implicates the nuclear matrix as an important architectural factor for the organization and possible function of FGFR1 in the cell nucleus. The nuclear matrix association of FGFR1 is also a constitutive property in these rapidly proliferating TE 671 cells, since the characteristic pattern of FGFR1 staining following nuclear matrix extraction was observed in >95% of the cells.

The spatial organization of FGFR1, within the nucleus appears strikingly similar to that observed with splicing factors. It is well documented that splicing factors are enriched in regions of the nucleus that have been called "nuclear speckles" based on their characteristic staining [Turner and Franchi, 1987; Fu and Maniatis, 1990; Spector et al., 1991]. Several essential and nonessential splicing factors, including those belonging to the SR-repeat containing family of proteins, are targeted to the speckles [Misteli, 2000]. In double labeling experiments we found a very high degree of overlap (>90%) between sites of FGFR1 and splicing factors at speckled domains. In contrast, we observed much lower associations between the smaller sites of FGFR1 staining and splicing factors in the nucleoplasm devoid of speckles (<20%).

In addition to their presumptive role in mRNA processing, several studies have addressed the relationship of speckled domains to transcription sites. At the electron microscopy level, the speckled sites are believed to correspond to clusters of interchromatinic granules or ICGs [Fakan and Puvion, 1980; Spector et al., 1991; Spector et al., 1993]. Due to an apparent lack of uridine incorporation as visualized with electron microscopic autoradiography, these sites were proposed to be storage compartments for splicing factors [Fakan and Nobis, 1978; Wansink et al., 1993; Gama-Carvalho et al., 1997; Cmarko et al., 1999]. However, several studies have documented the association of certain actively transcribing genes both on the periphery and within the nuclear speckles [Huang and Spector, 1991; Xing et al., 1993; Xing et al., 1995; Smith et al., 1999; Johnson et al., 2000]. Furthermore, both hyperphosphorylated RNA pol II and nascent transcripts labeled with BrUTP have been demonstrated to associate with speckles [Mortillaro et al., 1996; Patturajan et al., 1998; Wei et al., 1999].

Our results demonstrate a significant association of transcription sites and hyperphosphorylated RNA pol II with domains of FGFR1 staining. Both the large and small sites of FGFR1 were found to associate with sites of transcription or pol II (approx. 30–40% overlap). Among the large domains, sites of transcription and pol II were found on the periphery as well as in the interior domains of

FGFR1 staining. Moreover, the association seen with transcription sites is specific as demonstrated by a lack of colocalization observed between FGFR1 and sites of DNA replication. These results are consistent with an earlier report indicating the selective association of transcription but not replication sites with nuclear speckles [Wei et al., 1999].

The presence of transcription sites inside and on the periphery of speckles further suggests that, at least in some instances, the two processes of transcription and RNA splicing are coordinated within the same 3-D space of the nuclear speckle. Contrary to this "functional model," speckles could represent sites where splicing factors are concentrated in the nucleus prior to being recruited to active transcription sites [Misteli, 2000]. In this "storage model" of the speckle, the less intense diffuse staining of splicing factors observed throughout the nucleoplasm could correspond to this dynamic active recruitment of splicing factors to sites of ongoing transcription. Our results are consistent with both models. Indeed, the nonspeckle, smaller FGFR1 sites are associated with transcription sites and pol II at levels similar to that at the nuclear speckles. There is, therefore, no a priori reason why transcription/RNA splicing could not occur concurrently at both nuclear speckles and nonspeckled sites.

Further studies will clearly be needed to elucidate the functional and regulatory significance of nuclear FGFR1 localization at nuclear speckles versus the much smaller nonspeckled domains. Recently, it was demonstrated that activation of appropriate target cells with signals that induce translocation of FGFR1 into the nuclear interior results in an apparent recruitment of FGFR1 to sites of nuclear speckles [Peng et al., 2002]. Thus, FGFR1 following cell activation, shows an immunofluorescence labeling pattern over nuclear speckles [Peng et al., 2002] that is virtually identical to what is observed constitutively in the rapidly proliferating cells analyzed in this present study.

We, therefore, propose that nuclear speckles function as at least one of the compartments in the cell nucleus where programmed regulation of transcription and RNA splicing occur via FGFR1 mediated events. If this is the case, then it is possible that other signaling and regulatory factors which interact directly or indirectly with the FGFR signaling system are also components of nuclear speckles. Indeed, Yuan et al. [1998] demonstrated that a tyrosine phosphatase and Mortillaro and Berezney [1998] showed that a nuclear matrix cyclophilin which exhibited a protein folding related peptidylprolyl *cis–trans* isomerase activity are associated with nuclear speckles. Moreover, Tabellini et al. [2003] recently reported that several regulatory factors involved in the nuclear phosphoinositide signaling pathway of cells [Martelli et al., 2000; Cocco et al., 2002] are localized at the nuclear speckles. This raises the possibility that these compartments, aside from an obvious role in storage, are major *regulatory factories* in the cell nucleus where a multitude of signaling and regulatory factors can intersect dynamically to mediate reprogramming of transcriptional and cellular processes.

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