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Technical Advances

Detection of Epidermal Growth Factor Receptor mRNA in Tissue Sections from Biopsy Specimens Using *in Situ* Polymerase Chain Reaction

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Human epidermal growth factor (EGF) receptor mRNA was detected in cryopreserved tissue sections adherent to whole glass slides using* in situ *reverse transcriptase polymerase chain reaction. EGF receptor cDNA was synthesized* in situ *by reverse transcription using an EGF receptor-specific oligonucleotide primer.* In situ *polymerase chain reaction amplification in the presence of digoxigenin-11-dUTP and subsequent binding with an antidigoxigenin antibody conjugated to alkaline phosphatase allowed direct visualization. Because DNase, RNase, or proteinase K are not required, tissue integrity is maintained. EGF receptor mRNA is expressed in the basal layer of normal human skin epithelium and is significantly overexpressed in squamous cell tumor specimens, which is consistent with conventional analysis of EGF receptor expression. The assay is semiquantitative, quicker, more sensitive, and void of the nonspecific binding associated with* in situ *hybridization.* In situ *reverse transcriptase polymerase chain reaction using whole glass slides is ideally suited for detecting moderate to infrequently expressed transcripts in biopsy specimens. (Am J Pathol 1994, 144:7–14)

The polymerase chain reaction (PCR) is a powerful method that allows enzymatic amplification of minute amounts of DNA or mRNA sequences allowing identification of defined nucleotide sequences isolated from purified cell(s).[1] The approach is much more sensitive than *in situ* hybridization[2] allowing identification of a single copy gene in as little material as a single cell.[3] The products of the reaction can be detected by incorporation of radioactive ligands or indirectly through incorporation of nucleotide conjugates that can be recognized by immunodetection.

Until recently, PCR has been applied only to DNA or RNA extracted from cells. PCR amplification has been extended to preserved tissues adhering to microscopic slides.[4–7] The products of the *in situ* PCR amplification have been subsequently detected by *in situ* hybridization (PCR-ISH) and have been used to study viral infections where multiple copies of the nucleic acid segment to be amplified usually exist. PCR-ISH has been applied to detect human papilloma virus in cryopreserved and paraffin-embedded tissue sections from squamous cell carcinoma of the cervix[4] and to identify HIV in infected and uninfected cells.[8] These studies have used the amplification of the specific DNA sequence, but the sensitivity and specificity of detection is limited by the sensitivity and

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specificity of the hybridization probe and by the conditions of the hybridization reaction. Most of these studies have used pretreatment of tissue sections with proteases and DNase to improve the accessibility of the target nucleotide sequence and the specificity of the hybridization reaction. The pretreatment typically perturbs the cellular morphology and histology often obscuring identification of the cells that contain the amplified nucleotide segments.

The technique described in this report uses only *in situ* PCR amplification after reverse transcription (*in situ* RT-PCR) without subsequent *in situ* hybridization to identify mRNA expressed in specific cells. Spann, et al had used *in situ* PCR amplification with radiolabeled ligands to directly demonstrate viral sequences in individual cells.[7] Modifying their approach to use pieces of glass slides that fit directly into the PCR tube, coupling the reaction with reverse transcription and directly incorporating digoxigenin-conjugated 11-dUTP (dig-11-dUTP) during the PCR amplification *in situ*, we have demonstrated that specific mRNAs expressed can be identified in tissue sections.[9] In this study we have modified this assay to use whole glass slides and biopsy specimens. Under these conditions using specific primers for the epidermal growth factor (EGF) receptor, mRNA can be detected *in situ* without use of trypsinogen and DNase-improving preservation of tissue morphology. This method was used to investigate the cellular distribution of EGF receptor mRNA within normal human epidermal tissues, tumors, and cultured cell lines.

Materials and Methods

Tissue Preparation

Normal human skin and squamous cell carcinoma biopsy specimens were obtained during surgical procedures performed for diagnostic purposes. Specimens were immediately frozen in liquid nitrogen and stored at −70 C. Cell sediments from tissue culture cell lines A431 (vulval squamous carcinoma cells), EJ (transitional cell carcinoma), and NALM (B cell lymphoma) with known EGF receptor expression were similarly processed for control purposes.[10,11] Frozen tissue sections (6 to 8 μm) were placed on charged glass slides (ProbeOn Plus, Fisher Scientific, Cincinnati, OH) and stored at −70 C for at least 16 hours to ensure adequate bonding of the tissue section to the charged glass slides.

Slides containing tissue sections were fixed in 3% buffered paraformaldehyde (100 mM $NaPO_4$, pH 7.4, and 5 mM $MgCl_2$) for 5 minutes. Slides were subsequently washed three times in phosphate-buffered saline and in autoclaved demineralized distilled water (ddH_2O). These tissue sections were directly used in the reverse transcriptase reaction.

Oligonucleotide Primers

A 27 base, antisense primer (5′ AATATTCTTGCTGGATGCGTTTCTGTA 3′) commencing at base 4056 of human EGF receptor cDNA (GenBank #X00588)[12] and a sense primer consisting of 30 bases (5′ TTTCGATACCCAGGAAGGCACAGCAGG 3′) commencing at 3855 were designed to specifically amplify a 202-bp segment of EGF receptor cDNA (Yuan and FJH, unpublished observations). These primers are specific for EGF receptor mRNA.

In Situ *Reverse Transcription*

Reverse transcription reaction solution contained buffer (final concentration 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$); 25 μM dATP, dGTP, dCTP, and dTTP (Pharmacia/LKB, Piscataway, NJ); 1 mM DTT, either EGF receptor-specific oligonucleotide primer (100 nM) or no primer; 1000 U/100 μl M-MLV reverse transcriptase (GIBCO BRL, Gaithersberg, MD); 75 U/100 μl RNAsin (Promega, Madison, WI); and ddH_2O. The reverse transcription reaction solution (100 μl) was applied to each slide and covered with Parafilm (American Can Co., Greenwich, CT) to prevent evaporation. Slides were incubated at 37 C for 1 hour on the heating block of a programmable thermal controller (Gene Machine II, Scientific Plastics, Wessex/UK-USA). The Parafilm coverslips were floated off by immersion in ddH_2O.

In Situ *PCR*

The slides were dried and a circle approximately 1.5 cm in diameter was marked around the specimen using a Pap pen (Daido Sangyo Co, Ltd., Japan) (Figure 1). PCR solution containing buffer (final concentration 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$); 50 μM dATP, dGTP, dCTP, and dTTP (Pharmacia/LKB); 1 mM DTT; 100 pmol of sense and antisense EGF receptor primers; 5 U Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN); and 2.5 μM dig-11-dUTP (Boehringer Mannheim) in a total volume of 60 μl was applied to the tissue section. An identical circle was marked

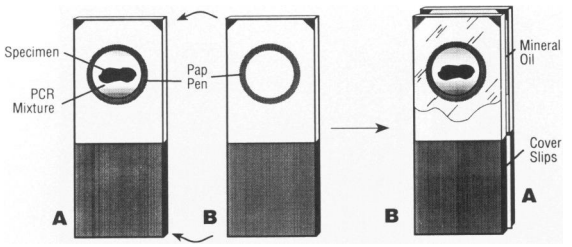

Figure 1. *Schematic representation of the slide preparation for the* in situ *PCR assay. Tissue sections adherent to ProbeOn Plus slides that had been fixed and reacted with reverse transcriptase according to Materials and Methods were dried and encircled with a PAP pen (slide A). An identical circle was drawn on either a blank slide or another slide containing tissue (slide B). The PCR reaction mixture (60 μl) was pipetted into the circle. One drop of mineral oil was placed on the frosted portion of slide A, between and on top of two coverslips (40 × 20 mm) that served as spacers. Slides A and B were placed face to face so that the circles touched and overlapped each other. Mineral oil was then pipetted between the two slides by capillary action encircling the PCR reaction mixture. The slides were subsequently placed on the surface of a thermocycler that had been covered with aluminum foil. The reaction was subsequently conducted according to Materials and Methods.*

on a second Probe-on Plus slide, which either contained additional tissue or was free of tissue. Two rectangular glass coverslips were placed at the frosted end of the slide to which the PCR solution had been placed. These coverslips increased the gap between the two slides. The two slides were placed face to face, trapping the PCR mixture within the Pap pen circles surrounding the specimen(s). Mineral oil was then introduced between the slides by capillary action to encircle the PCR solution to prevent evaporation. The slides were placed on the thermal cycler, the surface of which had been covered with aluminum foil. As many as 12 slides were amplified simultaneously.

The temperature on the surface of the slide during PCR was initially monitored and calibrated using a 1-mm thermocouple placed in mineral oil between two slides. The amplification reaction sequence involved denaturation at 94 C for 1 minute, primer annealing at 60 C for 1 minute, and extension at 72 C for 1 minute. The thermal cycler was programmed for the appropriate number of cycles with the final extension at 72 C for 12 minutes. The mineral oil was removed by capillary blotting before separation of the slides. Slides were washed twice in sodium citrate buffer (3 M NaCl, 0.3 M Na_3 citrate, pH 7.0, 2× SSC), 1× SSC, and 0.5× SSC for 15 minutes at room temperature with gentle agitation.

Immunological Detection

The digoxigenin-labeled cDNA segments were detected by enzyme-linked immunoassay using an antibody conjugate according to the recommendations of the provider (Boehringer Mannheim). An enzyme-catalyzed color reaction with 5-bromo-4-chloro-3 indolyl phosphate (x-phosphate) (Boehringer Mannheim) and nitroblue tetrazolium salt (Boehringer Mannheim) produced an insoluble blue precipitate that identified the location of the amplified EGF receptor cDNA molecules. The reaction with the chromogen was stopped by submersion in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). The reaction was stopped within 10 minutes or as soon as a deep blue-purple color was observed in any of the slides. Slides were not counterstained and binding was identified visually. The histological verification of the tissue that contained the amplified segments of EGF receptor cDNA was confirmed on duplicate sections stained with hematoxylin and eosin.

Results

In situ RT-PCR amplification of EGF receptor mRNA expression was studied in cryopreserved tissues from human tumor cell lines and normal skin tissue and squamous cell carcinoma biopsy specimens. To determine whether the assays were semiquantitative, sections from different tumor cell lines that are known to express a spectrum of EGF receptor detected on the cell surface by ligand binding assays and EGF receptor mRNA by Northern analysis and quantitative PCR were studied (Figure 2) (Yuan and FJH, unpublished observations)[10,13,14]. *In situ* RT-PCR in NALM-6 cells demonstrated no blue staining that would indicate EGF receptor mRNA, which have no detectable receptor. However, some cells within the pellet had an increased brown stain, which was secondary to the reaction with the chromogen. EJ cells that contain 1.86×10^5 receptor sites and 230 mRNA copies/cell showed an intermediate level of EGF receptor mRNA expression. A431 cells having 2×10^6 receptor sites and 2500 mRNA copies/cell had the most EGF receptor mRNA expression detected.

To demonstrate the specificity of the amplification, reverse transcription was performed in the presence of either antisense, sense primer, or no primer (Figures 3 and 4). Only reverse transcription in the presence of the antisense primer had been shown to synthesize the specific cDNA complementary to EGF receptor mRNA, which could be subsequently amplified by PCR.[9] The reaction with the sense primer was a control slide with the signal observing the result of both nonspecific cDNA synthesis during reverse transcription and genomic DNA

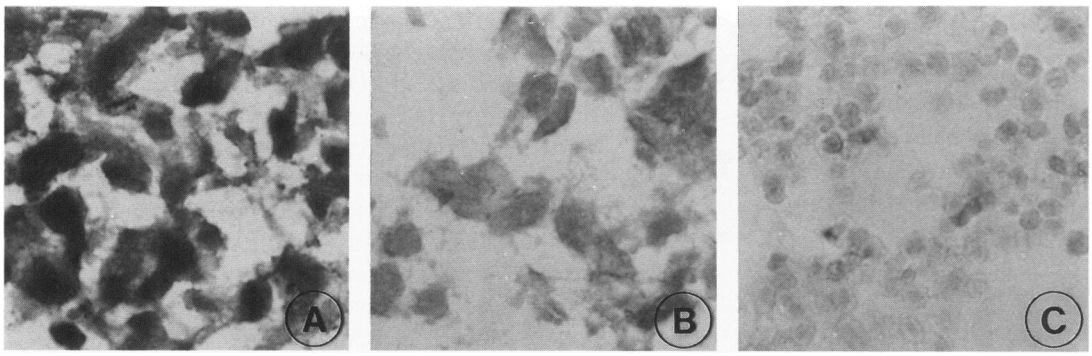

Figure 2. In situ *localization of EGF receptor mRNA by PCR in cell lines. Cryopreserved cell sections were obtained from cultured cells and assayed for EGF receptor mRNA expression according to Materials and Methods. The slides were not counterstained.* **A:** *A431 cells, squamous cell carcinoma of the vulva;* **B:** *EJ, transitional cell carcinoma;* **C:** *NALM-6, B cell lymphoma. The diffuse staining represents the intracellular localization of the EGF receptor cDNA (ie, mRNA) in* **A** *and* **B**. *In panel* **C** *some of the NALM-6 cells develop a faint nonspecific brown, but not the blue color due to the chromogen reaction. Magnification* ×*400.*

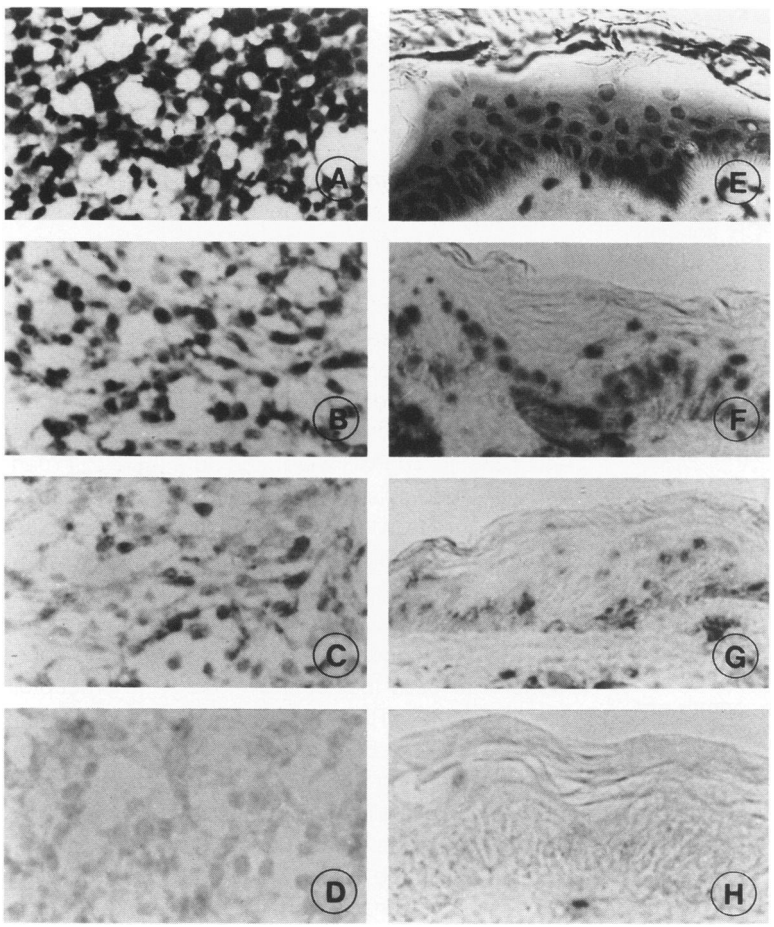

Figure 3. In situ *localization of EGF receptor mRNA in human epithelial tissues. Human cryopreserved epidermal tissues were either stained with hematoxylin and eosin* (**A**, *and* **E**) *or subjected to* in situ *RT-PCR followed by immunodetection of dig-11-dUTP incorporated into the amplified cDNA* (**B**, **C**, **D**, **F**, **G**, *and* **H**). *The dark staining in the lower six panels represents the localization of antidigoxigenin antibody conjugated to bacterial alkaline phosphatase.* **A:** *squamous cell carcinoma of the anus stained with hematoxylin and eosin;* **B:** *squamous cell carcinoma of the anus reacted with reverse transcriptase in the presence of the antisense primer;* **C:** *squamous cell carcinoma of the anus reacted with reverse transcriptase in the presence of the sense primer;* **D:** *squamous cell carcinoma of the anus reacted with reverse transcriptase in the absence of oligonucleotide primers;* **E:** *normal skin stained with hematoxylin and eosin;* **F:** *normal skin reacted with reverse transcriptase in the presence of the 3′ oligonucleotide antisense primer;* **G:** *normal skin reacted with reverse transcriptase in the presence of the 5′ sense oligonucleotide primer;* **H:** *normal skin reacted with reverse transcriptase in the absence of primers. B,* **C**, **D**, **F**, **G**, *and* **H** *were subsequently amplified by PCR for five cycles according to Materials and Methods. The diffuse dark cellular staining in* **B**, **C**, **F**, *and* **G** *represents cells that have amplified EGF receptor cDNA containing dig-11-dUTP. The staining is predominately nuclear.* **D** *and* **H** *have essentially no detectable dig-11-dUTP. Magnification* ×*200.*

during the PCR. The staining observed in the tissue sections treated without oligonucleotide primers during reverse transcription and subsequently subjected to PCR would result from amplification of single copy, genomic DNA. The amount of EGF receptor cDNA visualized in the tissue sections reacted with the antisense oligonucleotide primer during reverse transcriptase followed by fewer than seven PCR cycles was significantly greater when compared with tissue sections treated with the sense primer or in the absence of primer during the reverse transcriptase step. Amplified cDNA was de-

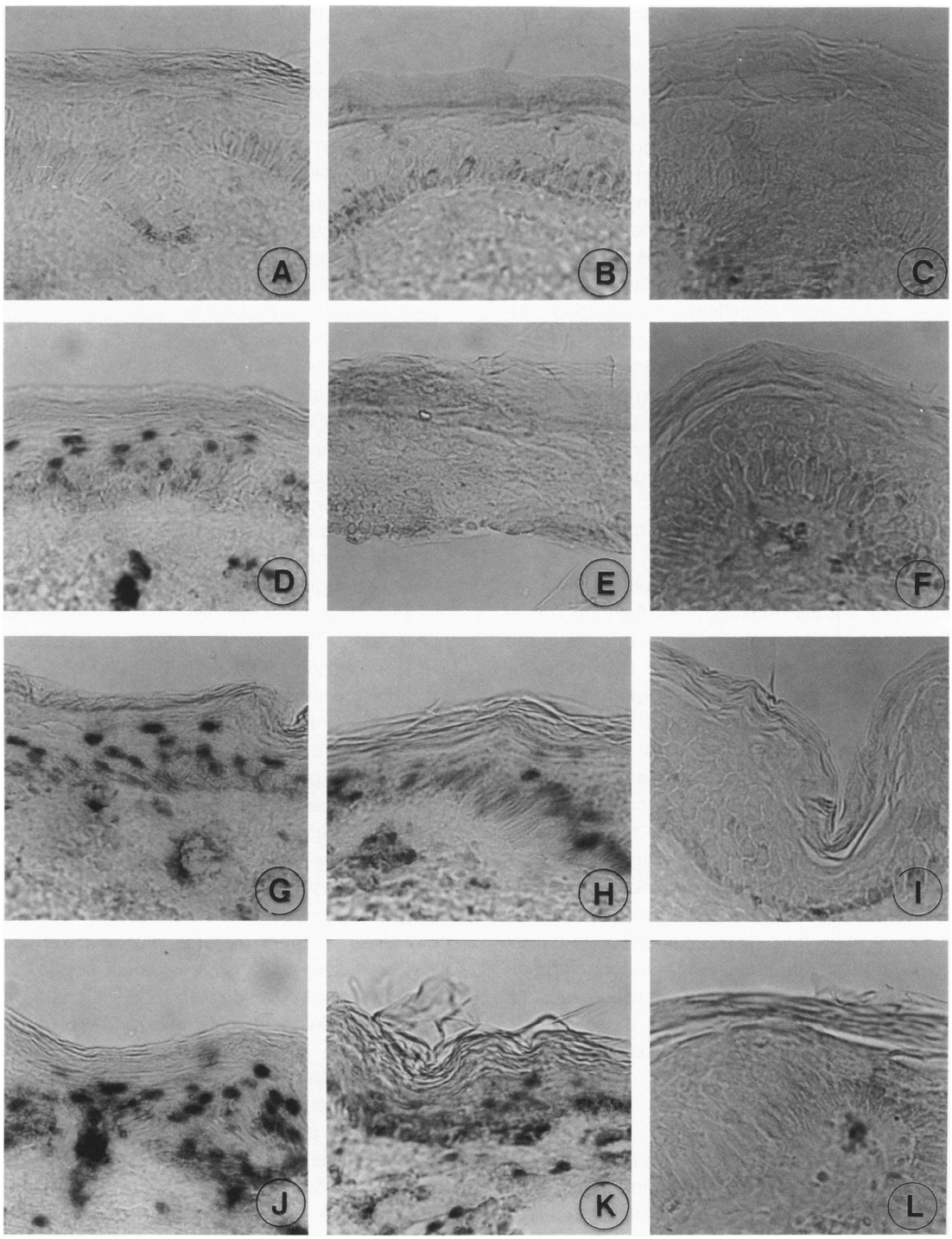

Figure 4. *The effect of cycle number on the detection of amplified cDNA. Normal human skin sections were reacted with reverse transcriptase in the presence of either antisense primer* (A, D, G, J), *sense primer* (B, E, H, K), *or no oligonucleotide primers* (C, D, I, L) *according to Materials and Methods. PCR was monitored for 1* (A, B, C), *3* (D, E, F), *5 and 7* (G, H, I), *10* (J, K, L), *15, 20, 25, and 34 cycles. The staining at the basal layer of the epithelium indicates the EGF receptor cDNA detected. Magnification ×100.*

tected in both control reactions after seven or more cycles of PCR. Pretreatment with DNase 1 reduced the staining detected in the absence of nucleotide primers in the reverse transcriptase portion of the reaction. Pretreatment with RNase free of DNase decreased the staining reaction with the antisense

primer as detected at the early cycles, but not that detected with the sense or the antisense primer after 10 or more cycles (data not shown).

To determine the optimal conditions for detecting the amount of EGF receptor mRNA being expressed, amplification was conducted from 0 to 35 cycles (Figure 4). EGF receptor mRNA was detected after 3 cycles (data not shown). The amount of EGF receptor mRNA detected in the assay of normal skin reached maximal values after seven cycles. The use of more than 10 cycles had no advantage. Additional amplification increased the level of staining observed in control sections, those reacted with the sense oligonucleotide primer, and those not reacted with primer during reverse transcription. The amplified products visualized in both of the control reactions increased after seven cycles eventually reaching a signal indistinguishable from that observed with the antisense primer. More cycles adversely affected tissue and cellular morphology.

In normal human skin EGF receptor mRNA expression was readily detected; the distribution was confined predominantly to the nuclei of cells in the basal layers of the epithelium. The EGF receptor mRNA observed decreased as keratinization occurred in the epithelial layer (Figure 3). In a squamous cell carcinoma of the anus, areas of increased mRNA expression correlated with the distribution of malignant squamous cells. The EGF receptor distribution was similar to that observed with EGF receptor antibody binding assays.[13,15] The pattern of staining observed in the control reactions after seven or more cycles was identical to that observed with antisense oligonucleotide primer (Figures 3 and 4). The staining in the control specimens was consistently greater in the tissue sections treated with the sense primer than those not exposed to primer during reverse transcription.

Discussion

In situ hybridization (ISH) has been used extensively in the past decade to study gene expression. Despite improvements in ISH, there still remain limitations in the sensitivity and specificity that affect utilization. In ISH the probe is labeled and the binding detected is that of the probe to the amplified sequences. The specificity and sensitivity is dependent on the degree to which the probe binds nonspecifically; the signal to noise ratio. Because the probe can bind to both DNA and RNA and nonspecifically to other cellular constituents, the tissue specimen must be treated with DNase and proteases. Larger probes tend to have difficulty entering cells, therefore, the tissue must be treated with proteases such as proteinase K, which can be detrimental to the cellular morphology and histology.

PCR-ISH offers significant advantages over ISH and *in situ* RT-PCR offers significant advantages over *in situ* PCR-ISH. *In situ* PCR amplifies infrequent nucleotide sequences within a cell, which can be detected and thereby increasing the sensitivity of the reaction. When this is coupled with ISH (PCR-ISH), then the identification is fraught with those reactions that affect the specificity of conventional ISH. With *in situ* RT-PCR, as described in this manuscript, the only nucleotides that will eventually be detected are nucleotides that are incorporated into the segments amplified by the PCR. Hence, there is essentially no nonspecific binding. If the PCR is specific, then the *in situ* amplification is specific. The specificity of the *in situ* reaction has been demonstrated by detection by agarose gel electrophoresis of only the specified sequence in the PCR mixture after greater than 30 cycles.[9]

The signal detected with the antisense oligonucleotide primer after no more than five cycles of PCR amplification appears to be due to EGF receptor mRNA synthesis because there is no signal detected in control sections. However, with greater than five cycles of amplification, signal is detected in tissues exposed to the sense oligonucleotide primer and the tissues reacted with no primer during reverse transcription-tissue sections, which cannot synthesize EGF receptor-specific cDNA during reverse transcription. Mild pretreatment with trypsinogen and subsequent nuclease digestion will not completely prevent reverse transcription and/or DNA amplification. This observation suggests that the EGF receptor mRNA and DNA within the tissue are not completely accessible to nuclease digestion after fixation. Extensive pretreatment with either RNase and DNase destroys the tissue section and obliterates the development of a signal even with the antisense primer.

Some of the non-mRNA sequences that are detected after five cycles are due to amplification of genomic DNA because signal is detected in tissue sections that have not been hybridized with primers during reverse transcription. The amplified sequences detected after five cycles with the sense primer are secondary to either nonspecific hybridization of primers during reverse transcription and/or amplification of genomic DNA (Figures 3 and 4). By selecting primers that hybridize specifically with mRNA, ie, primers that do not amplify genomic DNA, *in situ* DNA amplification can be avoided. If

this is not possible, the most effective method reducing the contribution from genomic DNA is to limit the number of PCR cycles. Signal secondary to nonspecific binding of primers to mRNA during reverse transcription can be reduced by increasing the temperature of the reaction to 50 C. Increasing the temperature of reverse transcription above 50 C decreased the signal detected.

The staining is predominately localized to the nucleus (Figure 3). Thus, the assay predominately detects nuclear mRNA. The unexpected predominance of nuclear staining may be the result of higher nuclear than cytoplasmic EGF receptor mRNA levels. Alternatively, cytoplasmic mRNA may be packaged and not accessible to the primers or the cytoplasmic mRNA may be more easily lost into the solution mixture during the PCR. The localization of the signal in control tissues cannot be discriminated from that observed with the tissues with the antisense oligonucleotide probe during reverse transcription. This observation has led us to speculate that *in situ* RT-PCR not only detects mRNA but under the proper conditions can identify transcriptionally active DNA.[9]

The assay appears to be semiquantitative (Figure 1). However, the number of amplified segments that remain adherent to the tissue sections is unknown. The extent of amplification achieved by PCR in solution is an exponential process.[1] The increment over the first seven cycles does not appear to be exponential as would be expected for a reaction on sequences in solution. Clearly, some of the amplified sequences remain adherent to the tissue but the intensity of the reaction no longer increases after seven cycles even when less dig-11-dUTP is used (data not shown). After 35 cycles using slides cut to fit into PCR tubes, EGF receptor mRNA can be detected in the PCR mixture.[9] The ability of the amplified product to remain adherent to the tissues is significantly affected by fixation and treatment with proteases. Performing the assay after pretreatment with trypsinogen[9] or other proteases reduces the signal detected.

The present assay is complementary to performing the assay on slides cut to fit into PCR tubes.[9] Performing the assay on tissue sections in tubes allows one to establish the conditions for the assay because the temperatures are identical to that developed during standard PCR amplification. When using whole slide only 12 slides can be studied simultaneously using up to six variable reaction mixtures, whereas with cut slides this limitation does not exist. Using cut pieces of slides limits the tissue section to no more than 2 mm. The whole slides allow study of tissues up to 1.5 cm in diameter; this is a major advantage when pathological specimens are studied in relation to adjacent normal tissues. Similarly, the greater area on the whole slide has allowed us to study multiple tissue sections adherent to the same slide avoiding any potential variation in experimental conditions. The preparation and use of slide pieces for the *in situ* RT-PCR assay is cumbersome. It requires cutting the slide to fit the tube, labeling the positively charged side for identification during tissue placement, and to avoid scratching the tissue during the many washes. The slides must be cut under conditions that avoid contamination with RNase. The slides are so small that it is difficult to properly place the tissue on the slide and many slides cannot be done quickly. Using the whole slide assay has allowed us to directly study cryopreserved sections from biopsy specimens. Cryosections can be taken directly from the surgical pathology laboratory at the time of diagnostic biopsy.

The technique described is novel using ProbeOn Plus slides sandwiched face to face for *in situ* PCR applications to human epithelial tissues. To our knowledge, this approach has not been previously applied to *in situ* PCR. The present method differs from others in several ways. The method uses only 60 μl of PCR reagents. By mounting two different specimens on the slides face to face, two slides can be reacted with the same PCR solution. Thus, duplicate and/or control specimens can be evaluated simultaneously. This technique decreases the amount of expensive reagents and increases the number of specimens that can be studied. The PAP pen and mineral oil seal the PCR reagents, preventing evaporative loss during temperature cycling and avoiding tissue loss often associated with glued-on coverslips.

The simultaneous incorporation of dig-11-dUTP label during PCR allows immunodetection and direct visualization of binding. The assay as described takes less than 36 hours. However, more quantitative data could easily be obtained by using radioisotope labeling of the PCR products and emulsion autoradiography with quantification by computer digitization. The nonisotopic method of detection used in the present protocol eliminates the hazards of radiation and further amplifies the sensitivity of the detection. Because digoxigenin is not a naturally occurring compound, *in situ* RT-PCR as described is as specific as when radiolabeled nucleotides are incorporated into the amplified segment. Single copy genes can thus be detected in normal human tissues (Figure 3). The assay is so sensitive that the amount of dig-11-dUTP has been

reduced to 1 of 20 of that recommended for matrix hybridization and the exposure time of the chromogen is reduced to no more than 10 minutes.

In situ RT-PCR appears to be universally applicable and is capable of detecting low copy mRNAs. The conditions of the assay are essentially those established in standard reverse transcriptase PCR with mRNA, cDNA, and genomic DNA in the reaction mixture. To avoid nonspecific hybridization of the oligonucleotide primers, the conditions of the reactions of both reverse transcription and PCR are as stringent as possible. Whenever possible we have used oligonucleotide primers that do not react with genomic DNA. Using this approach, we have successfully detected EGF receptor, transforming growth factor-α and -β, and *fli-1* mRNAs with *in situ* RT-PCR (FJH et al, unpublished observations).

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