Nucleic Acids Research, 1993, Vol. 21, No. 14 3159–3166

Variation in cellular EGF receptor mRNA expression demonstrated by *in situ* reverse transcriptase polymerase chain reaction

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Received April 9, 1993; Revised and Accepted May 19, 1993

ABSTRACT

Cell to cell variation of epidermal growth factor (EGF) receptor mRNA levels in heterogeneous tissues has been demonstrated with an *in situ* assay that couples reverse transcriptase with the polymerase chain reaction (*in situ* RT–PCR). EGF receptor mRNA is consistently more highly expressed in regions where cell division occurs; EGF receptor mRNA is markedly reduced if not absent in areas of squamous cell differentiation. Both human and mouse tumors overexpress EGF receptor mRNA when compared to normal tissue. *In situ* RT–PCR performed on thin sections obtained from cell pellets of cultured cells with known levels of EGF receptor mRNA expression demonstrated that the mRNA detected is consistent with that observed by Northern analysis and quantitative PCR on isolated RNA and by protein levels detected by antibody binding assays. *In situ* RT–PCR is significantly more sensitive than *in situ* hybridization (ISH). The method avoids background associated with hybridization reactions as in ISH or ISH following *in situ* PCR. *In situ* RT–PCR appears to be applicable to any gene as long as the oligonucleotide primers used have been proven to be specific and effective in a standard RT–PCR assay.

INTRODUCTION

Epidermal growth factor (EGF) receptor is expressed on the surface of normal epithelial tissue in association with dividing cells (1,2). EGF receptors have been shown to be consistently overexpressed in squamous cell carcinomas (SCC) of the respiratory tract (3). By Northern analysis the mRNA is increased in tumors (4,5) and the gene is amplified in at least 20% of respiratory tract SCC (3). We and others have observed an occasional lack of concordance between gene dosage and receptor number (3,6) indicating that it is necessary to evaluate mRNA expression in tissues.

EGF receptor expression on epithelial and tumor tissues is heterogeneous. In situ observations must be made to detect cell to cell variation. We had previously reported methodology to quantify EGF receptor expressed on the cell surface in situ (3). Here we report the detection of EGF receptor mRNA in situ using reverse transcriptase coupled with the polymerase chain reaction (in situ RT–PCR). This method builds on previous studies which used PCR amplification followed by in situ hybridization (ISH) to detect viral DNA in situ.

ISH has been used to detect EGF receptor mRNA expression in tumors when the mRNA is present in significant excess relative to normal tissue (7). However, we have been unable to consistently detect the level of EGF receptor mRNA in tissue sections of normal epithelial using either cDNA, cRNA or oligonucleotide probes in ISH because of the signal to noise ratio. Since monoclonal antibodies Pwhich recognize the mouse EGF receptor are not available, we needed a means of studying EGF receptor expression in mouse tissues. For these reasons an assay to identify EGF receptor mRNA in situ utilizing the polymerase chain reaction (PCR) was developed . Since PCR can detect single copy DNA within a single cell (8), it is superior to in situ hybridization (ISH) for detecting low copy numbers of DNA and infrequently expressed mRNA (9). PCR has been conventionally performed on DNA or cDNA obtained from tissue specimens, tissue sections, or whole cells with a loss of tissue architecture. Identification of cells or the proportion of cells synthesizing the targeted nucleic acid sequence can not be determined. Recently, in situ PCR amplification followed by ISH (PCR–ISH) has been used to detect viral DNA in host cells (10,11,12). The latter method appears to increase the sensitivity of ISH so that single copy DNA can be detected (9). PCR–ISH appears to significantly increase sensitivity while identifying the cells of interest. Spann et al have been able to obviate the need for ISH when detecting viral DNA in host cells by incorporating the label directly into the segment of DNA synthesized during the PCR (12). A preliminary report indicates that PCR–ISH can be used to detect intracellular mRNAs (9). Embleton et al have detected

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rearranged intracellular immunoglobulin mRNA in intact mouse hybridoma cells using nested fluorescent tagged primers in the PCR amplification (13).

In this report we have utilized the approach of Spann et al. for detecting viral DNA within cells (12) and have extended their method to assay EGF receptor mRNA in tissue sections. In situ RT−PCR successfully detects and localizes native intracellular EGF receptor mRNA sequences within cryopreserved tissue sections. The assay appears to be semi-quantitative. Differing levels of expressed mRNA can be discriminated in thin sections of cultured cell pellets and cryosections obtained from normal and malignant human and mouse tissues. We have demonstrated cell to cell variation of epidermal growth factor (EGF) receptor mRNA levels in heterogeneous tissues in situ. EGF receptor mRNA is consistently more highly expressed in regions where cell division occurs; EGF receptor mRNA is markedly reduced if not absent in areas of squamous cell differentiation. Both human and mouse tumors overexpress EGF receptor mRNA when compared to normal tissue. The assay appears to be applicable for identifying any nucleotide segment where the PCR primers have been established with DNA or cDNA in solution.

METHODS

Tissues and cultured cells

Human normal oral mucosa and head and neck SCC tissue specimens were obtained at the time of diagnostic biopsy. Normal mouse tongue and SCC of the tongue were obtained from CBA mice. The tumors had been produced by triweekly exposure to 4-nitroquinoline-1-oxide for 16 weeks (14). Tissues were immediately frozen in liquid N_2 and stored at −70°C. Human tumor cells lines, A431 (SCC of vulva), HN-5 (SCC of head and neck), EJ (transitional cell carcinoma of bladder), and NALM-6 (B-cell lymphoma) having known amounts of EGF receptor mRNA and receptor expression were grown in minimal essential medium with 5% calf serum to 75% confluence (3,4). Cells were harvested by scraping and centrifugation, washed with phosphate buffered saline (PBS), and immediately frozen in liquid N_2.

Preparation and fixation of specimens

Cryosections, 6 to 8 μm in thickness, were cut and placed on pieces of charged glass slides (*ProbeOn Plus* Microscope Slides, Fisher Scientific, Cincinnati OH) that had been cut to fit in 500 μl tubes to be used for thermocycling. The slides with the specimens were each placed in the tube in which they remained throughout the entire procedure. The specimens were stored at −70°C for at least 16 hours to improve adherence to the slide. Slides were stored for up to 4 weeks. Upon removal from the freezer, the sections were fixed in buffered 10% formalin overnight and washed three times in PBS and twice in autoclaved distilled demineralized water (ddH_2O).

Oligonucleotide primers

Oligonucleotide primers of 27 bases complementary to EGF receptor mRNA (anti-sense primer) which begins at base 4058 of human EGF receptor cDNA (15) with the sequence, 5′ AATATTCTTGCTGGATGCGTTTCTGTA 3′ and a 30 base oligonucleotide, 5′ TTTCGATACCCAGGACCAAGCCAC-AGCAGG 3′, with its sequence contained in the mRNA (sense primer) which begins at base 3856 (15) were used in the reverse transcriptase and PCR. These primers were chosen because they are highly conserved being identical in both human and mouse EGF receptor cDNA sequences (15,16). They are specific in standard PCR reactions for EGF receptor mRNA (Yuan and Hendler, unpublished data). When PCR amplification is performed with these primers, a 202 base pair segment of the EGF receptor cDNA is amplified which is just 3′ to the translated portion of the EGF receptor coding region.

Pretreatment of specimens

Tissue sections were permeabilized with 2 mg/ml trypsinogen (Sigma, St Louis MO) in 0.01 N HCl for 15 min at 25°C and neutralized with 0.1 M Tris HCl (pH 7.5), 0.1 M NaCl (11). RQ1 RNase-free DNase (8 U/100 μl) (Promega, Madison WI) in 40 mM Tris HCl (pH 7.9), 10 mM NaCl, 6 mM $MgCl_2$, 0.1 mM $CaCl_2$ at 37°C for 10 min was used to degrade the DNA. DNase was inactivated by heating to 75°C for 10 min.

Reverse transcription

Oligo $d(T)_{15}$ (Boehringer Mannheim, Indianapolis IN) or either EGF receptor oligonucleotide primer were used in the reverse transcriptase reaction. Reverse transcription was carried out in a total volume of 100 μl in each 500 μl tube containing a tissue section. The final concentrations for the reaction mixture were as follows: 10 mM Tris HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 25 μM deoxynucleotides [dATP, dCTP, dGTP, dTTP (Pharmacia, Piscataway NJ)], 1.2 μM oligo $d(T)_{15}$ or 100 nM of either EGF receptor primer, and 10 mM DTT in 100 μl. The reaction mixture contained as well 75 U RNasin (Promega) and 400 U M-MLV Reverse Transcriptase (GIBCO BRL, Gaithersberg MD). The specimens were incubated in the above reaction mixture for 1 hour at 50°C with oligo $d(T)_{15}$ and the EGF receptor primers. The solution was removed by aspiration and the slides were washed five times each in sodium citrate buffer [3 M NaCl, 0.3 M Na_3Citrate, pH 7.0 (2× SSC)], 1× SSC, 0.5× SSC and twice with ddH_2O.

Polymerase chain reaction

The PCR amplification was carried out in 100μl of 25 μM of each the nucleotides dATP, dCTP, dGTP (Pharmacia), 23.75 μM dTTP (Pharmacia), 1.25 μM digoxigenin-11-dUTP (dig-11-dUTP) (Boehringer Mannheim), 10 mM Tris HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 5 U/100 μl Taq DNA polymerase (Boehringer Mannheim), and 10 nM each of the sense and antisense primers. Prior to beginning the PCR, 90 μl containing all components of the reaction mixture except the nucleotides were added to the specimens and topped with 25−30 μl of mineral oil. The tube was heated to 95°C for five minutes in the thermocycler [Gene Machine (MJ Research, Inc.) or a Gene Machine II (Wessex, UK-USA Scientific Plastic)]. The temperature was decreased to 85°C for 5−10 minutes and the remaining 10 μl containing the nucleotides was added to each tube. The PCR amplification was carried out with denaturing at 94°C for 1.5 min, annealing at 60°C for 1 min, and primer extension at 72°C for 1 min except for the last extension which lasted 12 min. The PCR solutions were aspirated from each tube and saved in some experiments were examined by agarose gel electrophoresis (17). The slides while remaining in their tubes were rinsed in 500 μl of the following solutions, 2× SSC, 1× SSC, and 0.5× SSC, each 5 times at 25°C.

Detection of *in situ* RT−PCR amplified segments

Sections in the reaction vial were exposed to the PCR reagents for 70 cycles. Ten microliters of the reaction mixture was electrophoresed in 1% agarose containing 5 μg ethidium bromide/ml (17).

In situ hybridization

cDNA probe synthesis. A 202 base pair cDNA probe, identical to the fragment synthesized during the in situ PCR, was labeled with dig-11-dUTP in a standard PCR using the above described oligonucleotide primers except that the dig-11-dUTP was increased to 8 μM while the dTTP concentration remained constant.

Hybridization reaction. Cryopreserved tissue sections were fixed for 5 min with buffered 3% paraformaldehyde [100 mM $NaPO_4$ (pH 7.4), 5 mM $MgCl_2$] and washed with PBS. The tissue sections were digested by 1μg/ml proteinase K (Boehringer Mannheim) in 10 mM Tris HCl (pH 7.4), 2 mM $CaCl_2$ for 10 min at 37°C, 4 U/slide RQ1 RNase-free DNase (Promega) for 15 min at 37°C, and rinsed with PBS. DNase was inactivated at 65°C for 10 min and rinsed with ddH_2O. Slides were equilibrated for 30 min in 2× SSC and prehybridization mixture containing 50% formamide in 4× SSC, 1× Denhardt's, 500 μg single stranded salmon sperm DNA (Sigma Chemical), 250 μg tRNA (Boehringer Mannheim), 10% dextran sulfate (Sigma Chemical) at 42°C for 1 hr. The hybridization reaction was carried out in the same buffer containing the 202 EGF receptor cDNA, which had been denatured for 10 min at 95°C, for 16 to 18 hr at 42°C in the dark. Sections were rinsed twice with 2× SCC, 1× SSC, and 0.5× SSC at 25°C.

Immunodetection of PCR products

Immunodetection was performed according to the recommendations of the provider (Boehringer Mannheim). Briefly, the slides were equilibrated in maleate buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) for 5 min, incubated with 2% normal sheep serum (Sigma) and 0.3% Triton-X100 (Sigma) in the maleate buffer for 30 min and exposed for at least three hours at 24°C to 100 μl of anti-digoxigenin [Fab'] antibody conjugated to alkaline phosphatase (Boehringer Mannheim) diluted 1:500 in maleate/sheep serum/Triton-X100 buffer . The specimens were washed in maleate/sheep serum/Triton-X100 buffer twice for 10 minutes and subsequently washed in magnesium buffer (100 mM Tris HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 10 minutes. Five hundred microliters of chromogen [45 μl of nitroblue tetrazolium salt (NBT) solution and 35 μl of 5-bromo-4-chloro-3 indolyl phosphate (x-phosphate) (Boehringer Mannheim) containing 0.24 mg levamisole/ml (Sigma) of the magnesium buffer] was added to each tube. The slides were incubated in the absence of light for no more than 10 min in the in situ RT−PCR assay and for 6 hours in the ISH assay. The color reaction was halted with two washes of TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0). Tissue sections were not counterstained and were subsequently dehydrated in a graded series of ethanol dilutions and placed in xylene. Slide fragments were removed from their tubes and mounted on a coverslip. Binding was demonstrated microscopically by the presence of blue staining. To confirm the histologic identification of the tissues containing the EGF receptor mRNA, untreated tissue sections were fixed with 3% buffered paraformaldehyde and stained with hematoxylin and eosin.

RESULTS

To demonstrate the ability of in situ RT−PCR to detect cellular variation in mRNA expression, human cell lines known to express different amounts of EGF receptor were subjected to the assay using specific EGF receptor oligonucleotide primers. NALM-6, a B cell tumor cell line, which does not express EGF receptor on its cell surface and has no detectable EGF mRNA by quantitative RT−PCR (Yuan and Hendler, unpublished data) were negative by in situ RT−PCR (Figure 1). HN-5, a squamous carcinoma cell line with 1.5×10^7 receptor sites per cell and 3000 EGF receptor mRNA copies per cell, was strongly positive for EGF receptor mRNA in this assay. A431 (2.2×10^6 receptors/cell and 2500 mRNA copies/cell) and EJ cells (1.8×10^5 receptors/cell and 230 mRNA copies/cell) have intermediate mRNA as detected by in situ RT−PCR. A few EJ cells stain more intensely than the majority of cells. Some variability of staining is also observed in HN-5 and A431 cells.

To verify that the immunodetected segment was amplified from the cDNA product of reverse transcription and not from genomic DNA, tissue sections were hybridized with the antisense primer, the sense primer, oligo $d(T)_{15}$ or without any oligonucleotide primers in the reverse transcription step (Figure 2). Only the sections exposed to the antisense primer and oligo $d(T)_{15}$ should produce an appropriate cDNA. Any sequence amplified in the absence of primer would necessarily be of genomic origin (Figure 2d) or the result of a non-specific hybridization with the sense primer with subsequent synthesis of cDNA with reverse transcriptase (Figure 2b). Only the reverse transcriptase with the antisense primer or oligo $d(T)_{15}$ primer followed by 5 cycles of PCR with both primers produce a significant mRNA signal in the assay. There was no consistent difference in the mRNA detected in tissue sections following in situ RT-PCR with either the antisense or oligo $d(T)_{15}$. In contrast both the sense primer and the reverse transcriptase without primer followed by PCR produced little signal at 5 cycles.

There are a number of important parameters to be considered in the assay. The optimum number of amplification cycles necessary to detect the in situ RT−PCR product was determined using normal mouse tongue epithelium (Figure 3). At 1 cycle the slides were essentially negative, but by 3 cycles cells along the basement membrane are positive for expression of EGF receptor mRNA. The number of cells appearing positive and the intensity of the color reaction increased until 5−7 cycles at which the maximum signal was attained. The staining at 10 cycles appears to be less than that detected at 7 cycles. In normal skin

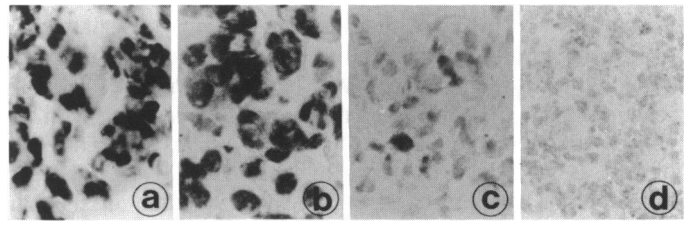

Figure 1. Immunodetection of EGF receptor mRNA in cultured cells. Cryopreserved tissue sections obtained from cell pellets of human cultured tumor cells were subjected to in situ RT−PCR according to the Methods using oligo $d(T)_{15}$ as the primer with RT. The PCR was for 5 cycles. Panel **a**: HN-5; **b**: A431; **c**: EJ; **d**: NALM-B. Magnification 200×.

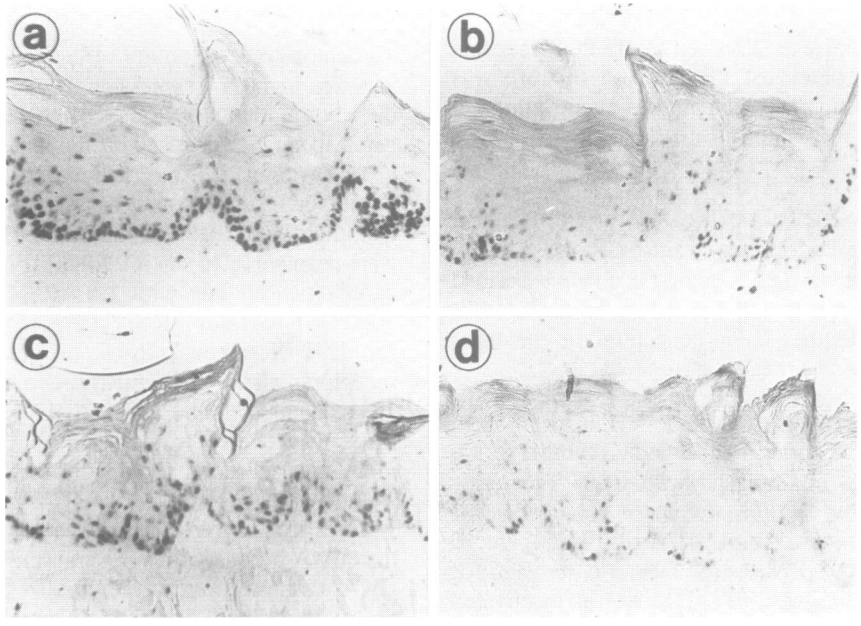

Figure 2. Verification of the specificity of in situ RT−PCR. Normal mouse tongue cryopreserved sections were treated with RT in the presence of either the EGF receptor antisense (panel **a**), EGF receptor sense primer (panel **b**), oligo $d(T)_{15}$ (panel **c**), or no primer (panel **d**). PCR amplification was for 5 cycles in the presence of both the sense and antisense EGF receptor primers. Magnification 80×.

tissues this was a consistent observation. The color reaction in the control tissue sections, sections hybridized to the sense primer or no primer and reverse transcriptase, increased more slowly becoming similar to the signal detected in the tissue section with the antisense primer by 10 cycles.

To demonstrate that the product amplified in the in situ reaction mixture was the target segment, tissue sections were amplified for 70 cycles. The appropriate 202 bp segment was consistently detected in the reaction solution of HN-5 cell pellet thin sections adherent to slide fragments by agarose gel electrophoresis (Figure 4). This segment when cut with Sca I yielded two bands of 88 and 114 base pairs. However, even after 70 cycles the amplified segment could not be consistently detected in the solution recovered from EJ, NALM, and normal mouse tongue tissue sections.

To compare in situ RT−PCR with ISH, similar cryopreserved sections were hybridized to the identical EGF receptor cDNA segment amplified in situ (Figure 5). The segment used for ISH had been labeled with dig-11-dUTP (1:3 dig-11-dUTP to dTTP) yielding a probe of approximately 5 times greater specific activity than the amplified segment produced in the in situ RT−PCR. The probe was mixed with the tissue sections under standard conditions for ISH. The conditions for the reaction with the antibody were identical to that used in the in situ RT−PCR reaction. However, to detect the signal, the reaction with the chromogen had to be increased to 6 hours. Thus, the sensitivity of ISH is at least two orders of magnitude lower. There is considerably greater background with ISH than in the situ RT−PCR.

Murine squamous tissue and squamous cell carcinoma specimens were examined using the same EGF receptor specific oligonucleotide primers. The reaction worked equally as well in human and mouse tissue (Figure 6). In both human and mouse tissues, the pattern of EGF mRNA detected is associated with the dividing cells. The level of intensity in the normal epithelium is greatest in the basal layer of the epidermal cells and decreases as differentiation occurs. There is very little if any EGF receptor mRNA detected in keratinized cells. In tumor sections the EGF receptor mRNA is expressed in greater amounts in association with the proliferating cell population and is decreased in the areas of differentiation (i.e. keratin formation). The level of EGF receptor mRNA is higher in both mouse and human tumors than the basal layers of normal epithelium.

DISCUSSION

This report demonstrates that PCR can be used to detect EGF mRNA in cryopreserved tissue sections, i.e. in situ. EGF receptor mRNA detected using in situ RT−PCR in human heterogeneous tissues demonstrates cell to cell variation that is similar although not identical to the distribution observed with EGF receptor antibody assays in situ which detect the EGF receptor on the cell surface (1,2). The EGF receptor mRNA distribution appears more limited to the basal layers of normal human skin than the receptor expressed on the surface. The EGF receptor expression demonstrated in this assay is consistent with that detected by northern analysis (4) and by quantitative RT−PCR on isolated tissue culture cells (Yuan and Hendler, unpublished data). In situ RT−PCR detects EGF receptor mRNA in both human and murine normal squamous epithelium and tumors. The mRNA expression is greatest in areas of cellular proliferation and is least in regions of squamous differentiation. EGF receptor mRNA expression is significantly higher in both the human and mouse squamous tumors. These results are consistent with the overexpression of the receptor that has been observed in human tumors.

The signal detected by in situ RT−PCR following 5 cycles of PCR is predominately nuclear. Similar observations have been

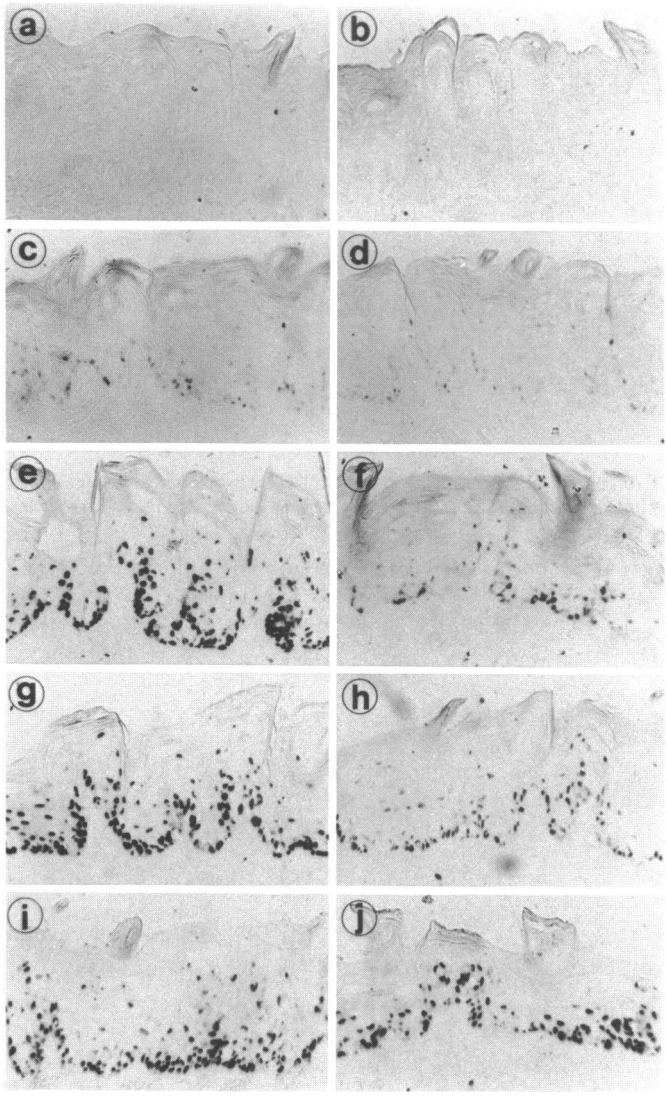

Figure 3. The effect of the number of thermocycles on immunodetection of EGF receptor mRNA. Normal mouse tongue cryopreserved sections were treated with reverse transcriptase according to the Methods in the presence of either the EGF receptor specific antisense (panels **a,c,e,g,i**) or sense oligonucleotide primer (panels **b,d,f,h,j**). PCR amplification was stopped after the following number of thermocycles—Panel a,b: 1 cycle; c,d: 3 cycles; e,f: 5 cycles; g,h: 7 cycles; i,,j: 10 cycles. EGF receptor mRNA was identified by immunodetection according to Methods. Magnification 80×.

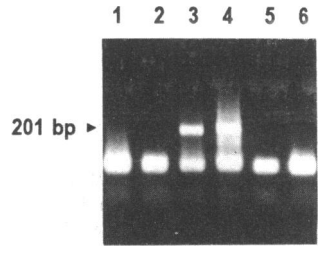

Figure 4. Demonstration of in situ RT−PCR products in the PCR mixture. Tissue sections adherent to pieces of slides were treated with reverse transcriptase, oligo $d(T)_{15}$, and dNTP.and the reaction mixture removed. The PCR mixture containing dig-11-dUTP was added to the slides and 10 μl of the mixture and 70 cycles of PCR was performed according to the Methods. The PCR products were electrophoresed on 1% agarose and stained with ethidium bromide. mRNA was identified in those tissue slides that were reacted with dig-11-dUTP by immunodetection. The amplified sequence is 202 base pairs as indicated by the location of arrow. Lane #1, 3, 4: PCR of thin sections of cell pellets; Lane #2, 5, 6: PCR of the reverse transcriptase solution. Lane #1 and 2: NALM-6 cells; Lane #3−6 HN-5 cells.

made with TGF-α and TGF-β and *fli*-1 (data not shown). In each case, control experiments have demonstrated that this signal can be differentiated from the signal resulting from genomic DNA amplification by limiting the cycle number. Cell lines and tumors with significant overexpression of EGF receptor mRNA display more cytoplasmic staining. Although unexpected, lower levels of EGF receptor mRNA in the cytoplasm than in the nucleus could actually reflect the biological situation, i.e. cytoplasmic mRNA half life in normal cells is short. Alternatively, this difference could be an artifact if either the nuclear mRNA were more accessible to the oligonucleotide primers during the reverse transcriptase reaction or if the cytoplasmic mRNA were more easily lost during tissue preparation. Regardless, the experiments using cultured cells with known amounts of EGF receptor mRNA demonstrate that in situ RT-PCR does yield signals that are reflective of the amount of mRNA in the cells.

The variable amount of signal detected in thin tissue sections of cultured cells (Figure 1) is expected because unequal portions of cells are present within the section. More variability occurs cryosections because of variable desiccation in the cryostat at the surface of the section affecting the true thickness within the section. Hence, the mRNA present and detected in a cell should vary with the portion of a cell present as well as with the amount of EGF receptor per cell. Similar variability is observed in all cytologic assays of tissue sections including ISH (Figure 5). It is also possible that some of the variation in detected signal might reflect differences in cellular accessibility of the nucleotide primers or other components of the PCR to the mRNA. The same is, of course, true for other in situ methods that utilize hybridization and/or PCR.

Sections treated with reverse transcriptase in the absence of oligonucleotide primers give little detectable signal following 5 or fewer cycles of PCR amplification. By 10 cycles the contribution from genomic DNA is substantial and not different from that observed in tissue sections exposed to the antisense nucleotide primer and reverse transcriptase. These data suggest that the signal detected with the sense primer or without primers is due to EGF receptor genomic DNA which was not digested by the DNase treatment. Increasing the amount of DNase, $\geq$16 U, resulted in a loss of signal with the antisense primer as well as the sense and no primer controls. This presumably results from incomplete inactivation of the DNase and subsequent digestion of the cDNA synthesized during the reverse transcriptase reaction. Using an oligonucleotide primer which crosses an intron and is incapable of amplifying genomic cDNA, reduces the signal detected in the control PCR, but not completely (data not shown). This suggests that the amplification observed in the control reactions is asymmetric and arithmetic. The amplification observed with the antisense primers would be symmetric. Since the signal is detected with antibody and subsequent exposure to chromogen, signal from genomic DNA can not be discriminated from that arising from mRNA once a significant level of genomic cDNA has accumulated.

The staining observed in control tissues after 7 or more cycles is very similar to that detected with the antisense probe at 3 or

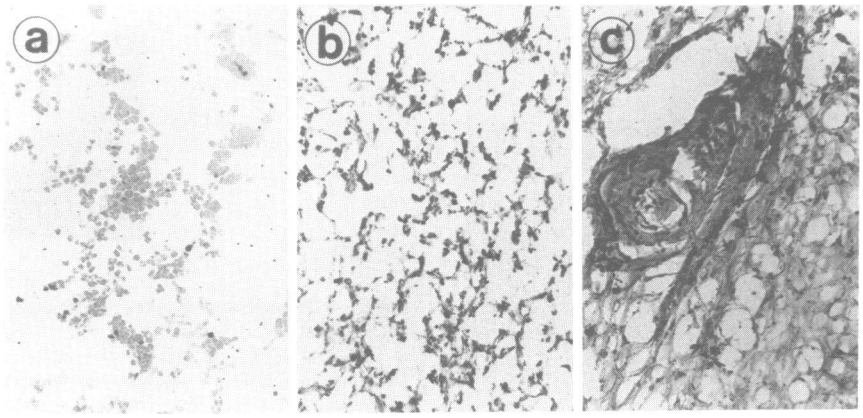

Figure 5. Detection of EGF receptor mRNA using in situ hybridization. The 202 bp EGF receptor cDNA labeled with dig-11-dUTP was hybridized to cryopreserved sections as described in the Methods. Panel **a**: NALM-6 cells; **b**: HN-5 cells; **c**: human nasopharyngeal SCC. The development of the alkaline phosphatase color reaction required 6 hours. Magnification 80×.

more cycles. Hence, the cDNA amplified in the control tissues appears quite 'specific'. Since no signal is detected in NALM cells which do not express EGF receptor mRNA (Figure 1d), it is our speculation that the only genomic DNA accessible to the oligonucleotide primers is transcriptionally active DNA.

In situ RT−PCR overcomes the inherent inability of standard PCR to identify the cell(s) synthesizing the nucleotide segment of interest. In situ PCR allows detection of target DNA or mRNAs within intact tissue and localizes the cell(s) that synthesize the amplified segment. The reaction is specific for the segment of cDNA or DNA that is amplified when appropriate conditions and primers are selected for the reaction. When Oligo d(T) is the primer for the reverse transcriptase, the specific antisense oligonucleotide primer in the PCR amplification acts as a nested primer and only the polyadenylated RNA will be synthesized as cDNA. If the target RNA is intact and always polyadenylated, the cDNA synthesized will reflect the amount of target RNA present. If the target RNA to be amplified is far from the polyadenylation site, then oligo d(T) primed cDNA are less likely to reflect the number of target mRNA present. Variation in polyadenylation would similarly decrease the amount of RNA detected. Hence, the EGF receptor primers used were directed to the 3′ untranslated region of the mRNA. However, if there were non-specific interaction of the sense primer to the EGF receptor mRNA during the reverse transcriptase reaction, non-target as well as target cDNA will be generated during the PCR. Since both contain a complementary sequence to the sense primer, they will be amplified during the PCR. The target cDNA will be amplified exponentially while the inappropriate, non-target cDNA arithmetically. Since the amplification is carried out for no more than 7 cycles, this is potentially a significant concern. For this reason we have increased the temperature of the reverse transcriptase reaction to 50°C with minimal loss of detectable mRNA and no evidence of significant non-specific cDNA formation (data not shown). Oligonucleotide primers which amplify genomic DNA under the conditions of the PCR will give a signal. The signal detected is insignificant in tissues that have single copy DNA when no more than 7 cycles of PCR are performed. However, in tumor tissue where EGF receptor genes may be amplified, the contribution from nuclear DNA could be significant. To overcome this problem we have reduced the cycle number to 5. This is significantly less than the 25 or more cycles used in ISH−PCR (9,10,11) or by Spann et al. (12).

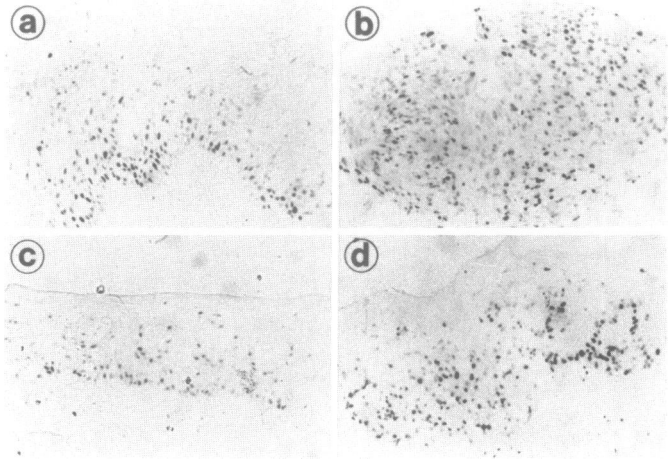

Figure 6. Immunodetection of EGF receptor mRNA in human and murine normal squamous tissue and tumors. Cryopreserved tissue sections obtained from human and murine normal and malignant squamous tissues were subjected to in situ RT−PCR according to the methods using the specific antisense EGF receptor oligonucleotide primer in the RT reaction. The PCR reactions was for 5 cycles. Panel **a**: normal human skin; **b**: human nasopharyngeal SCC; **c**: normal mouse tongue; **d**: mouse tongue SCC. Magnification 80×.

Non-specific incorporation of dig-11-dUTP can be assessed by exposing tissue sections to the sense primer during the reverse transcriptase reaction with subsequent production of cDNA during PCR. The contribution from genomic DNA can be determined by omitting oligonucleotide primers during the reverse transcriptase reaction.

In situ RT−PCR is more specific and sensitive than ISH when compared using cDNA labeled dig-11-dUTP (compare Figures 1, 5, and 6). The specific activity of the probe required to detect signal with ISH was 5 times greater than the amplified product synthesized with in situ RT−PCR and the exposure to chromogen was 60 times longer. Most investigators who have explored PCR in situ have utilized ISH to detect the amplified DNA (9,10,11).

Spann et al had demonstrated that this approach was unnecessary in detecting HIV infected cells with incorporated radiolabeled dCTP (12). In the present assay digoxigenin-11-dUTP is the labeled nucleotide incorporated during the amplification. Using immunodetection, there is further amplification and almost immediate detection of the cells with target cDNA. In situ RT−PCR is so powerful that the dig-11-dUTP was reduced to less than 1/20 of the recommended amount for synthesis of cDNA probes (Boehringer Mannheim) and the chromogen exposure reduced to no more than 10 minutes to discriminate positive from less positive and negative cells. Nuovo et al have demonstrated that ISH following PCR improves HPV detection 20 fold (9). With direct incorporation of dig-11-dUTP the present assay is significantly more sensitive than ISH and at least as sensitive as ISH−PCR.

There is no need for multiple primers to generate longer products to enhance detection (10). ISH−PCR and RT−PCR using tagged primers on whole cells (13) are subject to potential non-specific recognition of signal secondary to adherence of the labeled nucleotide probe. Decreasing the number of PCR cycles to 5, we have eliminated any significant signal from genomic DNA. By detecting only the product amplified from mRNA, avoiding the ISH step, there is little, if any non-specific signal detected. The only nucleotide segments that have the incorporated dig-11-dUTP are those that have been synthesized from the cDNA or genomic DNA. The only non-specific reactions which have to be avoided are recognition of unincorporated dig-11- dUTP, non-specific binding of the antibody, and inherent cellular alkaline phosphatase activity. These are clearly avoided under the assay conditions (Figure 1, 2, and 4). In addition by avoiding ISH, using dig-11-dUTP, and performing 5 thermocycles, the assay time can be cut to less 8 hours. Therefore, the use of ISH following PCR appears unnecessary.

Using in situ RT−PCT, EGF receptor mRNA can be readily detected after 3 thermocycles and is maximal by 7 cycles. At 10 cycles the intensity of staining is clearly decreased. Apparently, some of the amplification product is lost into the PCR solution but the exact number of amplified segments that remain attached to the tissue section is not known. The segment that we have amplified is 202 base pairs. By performing electrophoresis on the PCR reaction solution after more than 30 cycles of amplification, the desired nucleotide sequence can be detected but only consistently in the PCR reaction mixture from tissue sections that extensively overexpress EGF receptor mRNA. Production of only the expected nucleotide segment further verifies the specificity of the reaction. This verification is not possible when using ISH alone or in combination with PCR. The consistent loss of amplified product from tissue sections after 7 cycles indicates that any in situ assay of mRNA with more than 7 cycles of amplification is less quantitative than in situ RT−PCR.

The mechanism by which the amplified nucleic acids remains localized in the tissue section is unclear. If the amplified segments were not adherent in some way to the tissue, they should readily be detected in the reaction mixture, yet they are not. These results are somewhat in contrast to that obtained with poly(A) RNA adherent to an oligo(dT) matrix treated in a similar assay which results in consistent detection of the resultant PCR products in solution (18). We have increased the temperature of the melting reaction to 100°C and the time of melting to 3 min without improving our ability to detect the synthesized segment in solution and without effecting the in situ RT−PCR (data not shown). Some loss does occur and it becomes significant after 7 cycles since the amount of signal detected decreases consistently by 10 cycles (Figure 3). Since the products are not consistently detected in the reaction mixture, the leaking product must be loosely adhering to the tissue section and lost during the extensive washing procedure.

ISH−PCR (9,10,11) and labeled oligonucleotide primers (13) have been used predominately to study viral sequences or rearranged immunoglobulin mRNA for which there is no significant contribution from genomic DNA. If in situ RT−PCR only identifies transcriptionally active DNA, then control cell lines which do not actively synthesize mRNA would be negative controls in our assay. NALM-6 cells (Figure 1c), dermal cells, and mature and maturing keratinocytes (Figures 2, 3, and 6), which synthesize little if any EGF receptor mRNA, are negative. Obviously, in situ RT−PCR can be modified by selecting primers which react only with mRNA by crossing an intron. We have done this with EGF receptor and *fli*-1 where one primer crosses an intron (data not shown). Under these conditions signal still accumulates from genomic DNA. Presumably, this signal accumulates from the primer which can hybridizes to the genomic DNA. cDNA would accumulate asymmetrically and arithmetically. This cDNA can not be discriminated from appropriate double stranded cDNA because not enough signal accumulates to be characterized by gel electrophoresis. The genes which we have chosen to study do not have introns which would allow primers to cross both upstream and downstream introns. The assay as described herein works under properly controlled conditions, in the most difficult of all circumstances, where genomic DNA is present, transcription active, and no intron exists. This must be considered when comparing in situ RT−PCR with other in situ assays.

In situ RT−PCR works equally well in human and mouse tissue. The results observed with mouse tissue are similar to that observed in the human tissue. These results have not been demonstrated in mouse tumors heretofore because of an unavailability of antibodies which specifically recognize the mouse EGF receptor. It appears that the assay allows us to evaluate both human and mouse EGF receptor mRNA expression in situ. The assay demonstrates that EGF receptor mRNA is increased in 4-nitroquinoline-1-oxide induced mouse tumors further supporting the morphologic similarity with human head and neck tumors (14).

The present assay takes advantage of PCR conditions that were established using primers in a standard PCR mixture. The primers for EGF receptor had been shown to be specific for EGF receptor mRNA and to synthesize a segment which hybridize to both genomic and cDNA. The primers were selected because of their conservation in widely divergent mammalian species. The present report demonstrates that these primers can identify DNA and mRNA in both human and mouse tissue and presumably any other species where these sequences are conserved. The assay has been extended to detect TGF-α, TGF-β_1, and *fli*-1 mRNA without significantly altering the conditions established for PCR (data not shown). Thus, the method appears applicable to a wide variety of nucleic acid probes and is an attractive alternative to ISH for in situ analysis of nucleic acid expression.

ACKNOWLEDGEMENTS

Supported in part by the Department of Veterans Affairs, James Graham Brown Cancer Center Foundation, Alliant Community Trust, and Norwich-Eaton Resident Award.

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