

LOCALIZATION OF LATENT ADENOVIRUS INFECTION IN HUMAN  
LUNGS AND LYMPH NODES BY *IN SITU* PCR

BY

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## Abstract

Cigarette smoking is a major risk factor for chronic obstructive pulmonary disease (COPD), but only 15 to 20% of smokers develop airways obstruction. Respiratory infection caused by group C adenoviruses is a possible independent risk factor for COPD. Our working hypothesis is that adenoviral E1A DNA persists in airway epithelial cells following respiratory infections and is capable of amplifying cigarette smoked-induced airway inflammation. To develop a protocol that has the potential to detect low copy numbers of adenovirus E1A DNA in histological preparations of human lungs and lymph nodes, we first optimized *in situ* amplification of E1A DNA and subsequent detection of the DNA by *in situ* hybridization (indirect *in situ* PCR) on cytopsin and paraffin embedded preparations of Graham 293 cells which are known to have 4 to 5 copies of adenoviral E1A gene per cell. Using optimal conditions established for this indirect *in situ* PCR on paraffin embedded sections of Graham 293 cells, this procedure was performed on paraffin embedded sections of guinea pig lungs 20 days after the resolution of an acute infection with adenovirus 5 when no replicating virus could be recovered from these lungs (Vitalis, et al., 1996) and lungs and lymph nodes from COPD and non-COPD patients. For successful indirect *in situ* amplification of E1A DNA in Graham 293 cells, a "hot start" technique with 2 mM MgCl<sub>2</sub>, 1.5 μM E1A primers and 30 cycles of amplification was used. Application of indirect *in situ* PCR on cytopsin preparations of Graham 293 cells after pretreatment with 50 μg/ ml proteinase K for 5 minutes at 37°C resulted in nuclear staining in approximately 60% of the cells, while paraffin embedded 293 cells that had been digested with 1 mg/ ml pepsin in 0.2 N HCl at room temperature exhibited nuclear staining in approximately 40% of the cells. Staining was not seen in uninfected A549 cells nor in Graham 293 cells hybridized with an irrelevant probe or when Taq polymerase was omitted during amplification. Indirect *in situ* PCR on paraffin embedded sections of latently infected guinea pig lungs revealed

nuclear staining in bronchiolar and type II alveolar epithelial cells. Nuclear staining was also observed in alveolar epithelial cells when indirect *in situ* PCR was performed on paraffin embedded sections of lungs from COPD patients. As a comparison, direct *in situ* PCR, where labeled nucleotide is incorporated during amplification, was performed on cytopsin and paraffin embedded sections of Graham 293 cells using optimal conditions established for indirect *in situ* PCR. Our preliminary results from direct *in situ* PCR revealed significant problems of nonspecific signals. Our findings indicate that indirect *in situ* PCR allows the detection of 4 to 5 copies of adenovirus E1A DNA in Graham 293 cells. Localization of adenovirus E1A DNA in alveolar epithelial cells could have important implications regarding the regulation of proinflammatory agents that mediate neutrophil migration into the alveolar walls.



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## Introduction

Cigarette smoking is a major risk factor for the development of chronic obstructive pulmonary disease (COPD) (Fletcher and Peto, 1977), but only 15 to 20 % of smokers develop COPD even though all smokers show evidence of airways inflammation (Fletcher et al., 1976). There is considerable evidence suggesting that viral respiratory illness may also predispose patients to chronic airflow disorders (Becroft, 1967, 1971; McFarlane and Somerville, 1957). MacFarlane and Somerville (1957) showed serologic evidence of adenovirus infection in lobectomy specimens removed for bronchiectasis. Adenovirus was also isolated from lung tissue of children with an extensive necrotizing bronchitis and bronchiolitis and in these cases, bronchiolitis was considered to be a direct consequence of necrotizing lesions observed in the acute stage of disease (Becroft, 1967). Group C adenovirus (serotype 1, 2, 5, 6) are of particular interest because following acute infections, virus can persist in lungs, tonsils, and peripheral blood lymphocytes possibly as a form of a latent infection (Evans, 1958; Green et al., 1979; Horvath et al., 1986). Studies from our laboratory (Vitalis et al., 1997) showed that in a guinea pig model of latent adenovirus 5 infection 20 days after the resolution of the an acute infection both viral E1A DNA and the bronchiolitis caused by the virus persisted. Interestingly, the inflammatory response to a single acute exposure to cigarette smoke was enhanced in these latently infected animals (Vitalis et al., 1997). Furthermore, recent studies suggest that ICAM-1 expression is increased in the airway inflammatory process associated with cigarette smoke induced chronic airway obstruction (Gundel et al., 1992; Di Stefano et al., 1994).

The present study is based on the hypothesis that adenovirus E1A DNA persists in airway epithelial cells following viral respiratory infection and is capable of amplifying cigarette smoke induced airway inflammation. Adenovirus DNA has a linear double stranded structure (Younghusband and Bellett, 1971). The genome of adenovirus 2 and 5 is about 36000 base pairs



(bp) long (Sussenbach, 1984). The adenovirus genome is subdivided into early (E) and intermediate genes (I) which are expressed before the onset of viral DNA replication and late genes (L) which are transcribed after the replication of the viral DNA has started (Sussenbach, 1984). Electron microscopy *in situ* hybridization experiments suggest that the replication and transcription of adenovirus DNA takes place within the nucleus of the host cell (Puvion-Dutilleul and Puvion, 1991; Besse and Puvion-Dutilleul, 1994). Following adenovirus infection of permissive cells, the earliest transcripts detected map to the E1A region (early gene) of the genome (Sussenbach, 1984). The E1A region of adenovirus codes for two proteins of 243 and 280 amino acids that are expressed from two differentially spliced mRNAs of 12S and 13S, respectively (Boyd, et al., 1993). Both proteins can immortalize cells and cooperate with other viral and cellular oncogenes in the transformation of primary cells (Ruley, 1983). In addition, both proteins are involved in transcriptional activation and repression of several viral and cellular promoters (Boyd, et al., 1993). The mechanism by which E1A contributes to the pathogenesis of COPD is not clear, but E1A could play a role in the amplification of the inflammatory response by regulating activities of several cellular transcription factors that might be responsible for the expression of proinflammatory mediators (Whyte et al., 1988; Bandara and La Thangue, 1991; Chellappan et al., 1991; Keicho et al., 1997). Previously, we demonstrated that the presence of E1A induces ICAM-1 and IL-8 expression in the lung epithelial cells after their stimulation with LPS (Keicho et al., 1997a, 1997b). Recent studies also showed that E1A-mediated upregulation of these inflammatory mediators may be due to a common transcription factor, NF- $\kappa$ B, that is activated by E1A protein (Keicho et al., submitted for publication). Another possible mechanism of E1A-mediated enhancement of inflammation in lungs of COPD patients may be due to the ability of adenovirus to establish latent infection in lymphoid tissues and peripheral blood lymphocytes. Metcalf (1996) showed that in T-lymphocyte (Jurkat) and monocyte (THP-1) cell

lines transfected with an E1A expressing plasmid, E1A increased the activity of tumor necrosis factor (TNF) promoter compared to cells transfected with control plasmid. Promoter activity was increased further after PMA stimulation of Jurkat cells and LPS stimulation of THP-1 cells. This increase was reflected in an increase in TNF mRNA production after LPS stimulation. In other studies, guinea pigs latently infected with adenovirus infected a second time with E1A deleted adenovirus no longer capable of replication resulted in an inflammation that might be based on a host immune response (Vitalis, et al., submitted). Furthermore, other studies of guinea pigs latently infected with adenovirus showed that there is a significant increase in the CD4+ lymphocytes in both the airways and lung parenchyma after an acute exposure to cigarette smoke (Vitalis et al., 1997 and in press). The authors suggest that in these animals the increase in CD4+ cells may be due to clonal expansion triggered by a population of latently infected antigen presenting cells (i.e. dendritic cells) that are located in the subepithelium of the conducting airways and in the alveolar walls. An alternative explanation for the accumulation of CD4+ cells at the sites of chronic inflammation is that they were recruited from the peripheral blood due to the expression of adhesion receptors and release of various of cytokines such as IL-1 and TNF- $\alpha$  (Issekutz et al., 1994).

Although it has been shown that adenovirus persists in the lymphoid tissues and peripheral blood lymphocytes, previous studies from our laboratory on human and latently infected guinea pig lungs indicate that epithelial cells are the major site in which adenovirus persist (Elliott et al., 1995; Vitalis et al., 1996). Evidence for this includes the demonstration of E1A protein in alveolar and airway epithelial cells using immunohistochemistry. However, in lungs from COPD patients and in latently infected guinea pig lungs adenovirus DNA could not be detected by standard *in situ* hybridization, but the E1A DNA of adenovirus was readily detected by PCR followed by Southern hybridization (Matsuse, et al., 1992; Vitalis et al., 1996).

The experimental evidence that adenovirus E1A contributes to the pathogenesis of COPD in smokers is based on PCR results that the adenovirus E1A DNA was present in greater amounts in lungs of heavy smokers with airway obstruction than in controls with normal lung function matched for age, sex and smoking history (Matsuse, et al., 1992). However, one of the major drawbacks of PCR is that it requires nucleic acid extraction, which destroys tissue morphology, so that a correlation between E1A DNA and histological cell type(s) is not possible. *In situ* PCR is a technique that allows one to detect nucleic acids present at levels undetectable by standard *in situ* hybridization and correlate the result with morphology (Nuovo et al., 1991; Komminoth and Long, 1993; Bagasra et al., 1993). Localization of low-copy target DNA can be achieved by two different protocols for *in situ* PCR. In the direct *in situ* PCR technique, labeled nucleotides are directly incorporated into the PCR generated amplified products, permitting the non-isotopic detection of *in situ* amplified sequences whereas, indirect *in situ* PCR is performed with unlabeled nucleotides and the amplified products are detected by subsequent *in situ* hybridization using specific labeled probes (Long et al., 1992). The present study was undertaken to determine the specific cell types (i.e., epithelial cells, dendritic cell and lymphocytes) that harbor low copy numbers of E1A DNA in lungs of COPD patients using these *in situ* PCR techniques. In order to develop a protocol which would allow us to detect low copy numbers of adenovirus DNA in histological preparations of human lungs, Graham 293 cells (transformed human embryonic kidney cells) which are known to have 4 to 5 copies of adenovirus E1A DNA per cell (Graham, et al., 1977) and guinea pigs that had been latently infected with adenovirus 5 (Vitalis, et al., 1996) were used as model systems.

## Materials and Methods

### Cytospin Preparations for *in situ* PCR

Of all the *in situ* PCR protocols developed to date it appears that methods using intact cells (cytospins) provide optimal physical conditions for *in situ* DNA amplification (Long, et al., 1992). Compared to paraffin embedded sections, nucleic acids and membranes are better preserved in whole cells. Except for membrane permeabilization after an initial treatment with proteinase K, the membrane and nucleic acids in cytospins remain intact, whereas in paraffin embedded sections, after embedding and sectioning, both the nucleic acids and membranes are damaged. Also, the fixed whole cells appear to function as amplification sacks with semipermeable membranes that permit the primers, nucleotides, and DNA polymerase to pass into the cell and nucleus, yet sufficiently retard the outward diffusion of the larger PCR product to allow their *in situ* detection (Komminoth and Long, 1993). For these reasons, although the ultimate goal of this study was to localize the adenovirus E1A DNA in specific cells in paraffin embedded human lung and lymph node tissues, *in situ* amplification was tested initially on cytospin preparations. Whole cell cytospin preparations of Graham 293 cells (American Type Culture Collection, USA) which harbor 4-5 copies of adenovirus E1A DNA were used as a model system to determine whether *in situ* amplification of target DNA was feasible. Graham 293 cells grown in Eagle's minimal essential medium (Gibco BRL), supplemented with horse serum were removed from the culture flask by scraping off the plate and collected in a 15 ml centrifuge tube. A cell pellet was made by centrifugation at 1000 rpm for 5 minutes. The cells were washed in PBS (149 mM NaCl, 12 mM Na<sub>2</sub>HPO<sub>4</sub>, 4 mM KH<sub>2</sub>PO<sub>4</sub>) and then suspended in the same solution (1 x 10<sup>6</sup> cells/ml). A total of 100 µl of cell suspension was cytocentrifuged at 1500 rpm for 4 minutes onto silanized glass slides (1 x 10<sup>5</sup> cells/spot), air dried and stored at -20°C until needed. A549 cells (human lung carcinoma cell line that does not carry adenovirus

DNA) obtained from American Type Culture Collection (Rockville, USA) and grown in Eagle's minimal essential medium supplemented with 10 % fetal bovine serum (Hyclone, Logan, UT) were used as a negative control for *in situ* PCR and *in situ* hybridization. Cytospins of A549 cells were prepared as described for Graham 293 cells. A549 cells grown to confluence and then infected with adenovirus type 5 (American Type Culture Collection, Rockville, USA) for 24 hours were used as a positive control for *in situ* hybridization. *In situ* hybridization with adenovirus genomic probe (Enzo Diagnostics) showed nuclear staining in approximately 90% of these infected cells. To avoid the spread of infected air-borne viral particles during cytocentrifugation in the laboratory, adenovirus 5 infected A549 cells were fixed in 10% buffered formalin for 10 minutes and then cytospin preparations were prepared as described for Graham 293 cells.

## **Paraffin Embedded Sections**

### ***Graham 293 cells***

Graham 293 cells were grown to confluence, removed from the culture plate and pelleted as described above. The cell pellet was then fixed in 10% buffered formalin for 24 hours. Following fixation, the cells were washed in PBS to remove formalin and resuspended in PBS. A total of 2 ml ( $4 \times 10^6$  cells/ml) of these suspended cells were mixed with an equal volume (1:1) of 2 % low melting agarose and the agarose blocks were embedded in paraffin. Two 4  $\mu$ m-thick serial sections from the paraffin blocks were placed on silanized glass slides and stored at -20° C until needed. Paraffin blocks of uninfected and adenovirus 5 infected A549 cells were prepared as described for the Graham 293 cells. Histological sections of these paraffin embedded cells were prepared as described above.

### ***Guinea pig lung tissues***

Two blocks of paraffin embedded lungs from two latently infected guinea pigs that were previously studied in our laboratory (Vitalis, et al., 1997) were used as an animal model of latent adenovirus infection. DNA extracted from these lungs were positive for E1A by previous PCR and Southern blotting but were negative by standard *in situ* hybridization (Vitalis et al., 1997). Pairs of 4  $\mu$ m-thick serial sections from each block were placed on 8 silanized slides. The first 5 slides from each block were used for hybridization with biotinylated E1A probe after E1A *in situ* amplification. The next 2 slides were used for hybridization with an irrelevant probe after *in situ* amplification. The remaining 1 slide from one block was used for direct *in situ* PCR.

Two blocks of paraffin embedded lungs from two uninfected guinea pigs that were previously negative for E1A DNA by PCR and Southern blotting (Vitalis et al., 1997) were used as the negative controls. Pairs of 4  $\mu$ m-thick serial sections from each block were placed on 3 silanized slides and stored at -20° C until needed. The first 2 slides from each block were used for hybridization with biotinylated E1A probe after E1A *in situ* amplification. The remaining 1 slide from one block was used for direct *in situ* PCR.

### ***Lung and lymph node tissues from COPD patients***

Three blocks of paraffin embedded human lungs from three patients with chronic obstructive pulmonary disease who had undergone lung resection for cancer were available from our previous study (Matsuse et al., 1992). The procedure for fixation and embedding was described previously (Matsuse et al., 1992). DNA extracted from the lung tissue of these patients were positive for E1A DNA by previous PCR and Southern blotting, but were negative by *in situ* hybridization (Matsuse, et al., 1992). Also three blocks of lymph nodes were available from these patients, but these had not been tested for E1A DNA by PCR and Southern blotting. Pairs of 4  $\mu$ m-thick serial sections from each block of lung or lymph node were placed on 4 silane coated

glass slides and stored at -20°C until needed. The first 2 slides from each block were used for hybridization with biotinylated E1A probe after E1A *in situ* amplification. The next slide from each block was used for hybridization with an irrelevant probe after E1A *in situ* amplification. The remaining slide from one block of lung was used for direct *in situ* PCR.

#### ***Lymph node tissue from non-COPD patients***

Three blocks of paraffin embedded lymph nodes from three non-COPD patients who had lung resection for cancer were also available from a previous study. The procedure for fixation and embedding was described previously (Matsuse, et al., 1992). DNA extracted from the lymph nodes of two patients tested positive for E1A DNA by previous PCR and Southern blotting, while the lymph node from the third patients was negative for E1A DNA. Pairs of 4 µm-thick serial sections from each block of lymph node were placed on 3 silane coated glass slides and stored at -20°C until needed. The first 2 slides were used for hybridization with biotinylated E1A probe after E1A *in situ* amplification. The remaining 1 slide was used for standard *in situ* hybridization.

#### **PCR Primers**

The PCR primers used to amplify the E1A region of adenovirus 2 and 5 genomes specify 484 and 486 bp (base pair) products, respectively. The selected adenovirus E1A primers have the following sequence:

5'-TAATGTTGGCGGTGCAGGAAGG-3'

5'-TCAGGCTCAGGTTTCAGACACAG-3'.

The primers were tested initially by solution phase PCR on genomic DNA of adenovirus 2. The PCR reaction mixture (50 µl) contained 10 mM Tris buffer (pH 8.4), 50 mM KCl, 2 mM MgCl<sub>2</sub>, 0.001% gelatin, 200 µM dNTP, adenovirus 2 DNA (10<sup>5</sup> copies) and 0.5 µM of each

primer. After an initial denaturation step at 94°C for 2 minutes, 2 units of Taq polymerase (Gibco BRL) was added. Then, 40 cycles were performed on the RoboCycler (Stratagene) under the following conditions: denaturation at 94°C for 1 minute, annealing at 63°C for 1 minute and extension at 72°C for 2 minutes. The specificity of amplification was determined both by the size of PCR product on agarose gel electrophoresis and by subsequent Southern hybridization.

## Probe Preparation

The probe for the E1A region was a 756 bp product of a double digest with *Pst*I and *Bam*HI of a 742 bp *Alu*I fragment from pXC-15 (gift from Dr. Shenk, Princeton) containing the E1A region of adenovirus 2, which was subcloned into *Hinc*II site of pUC13 (Matsuse et al., 1992) and covers the entire sequence of the amplification product. The 756 bp fragment was purified by low-melting agarose gel electrophoresis and labeled with Bio-11-dUTP (Enzo Diagnostics) by the random priming method (Feinberg and Vogelstein, 1983). The 50 µl reaction mixture contained random priming buffer (50 mM Tris, 5 mM MgCl<sub>2</sub>, 10 mM DTT, 200 mM Hepes pH 6.8), 2 mg/ml bovine serum albumin (BSA), 72 µM each of dATP, dGTP, dCTP, 54 µM dTTP, 18 µM Bio-dUTP, 0.4 µg/µl random hexamer, 25 nanogram of purified 756 bp E1A DNA probe, 5 units of Klenow fragment (Gibco, BRL) and appropriate volume of water. As a control for non-specific *in situ* hybridization the 736 bp *Taq*I fragment from pUC13 was also labeled with Bio-11-dUTP by the random priming method. Both probes were then purified from the unincorporated Bio-11-dUTP and recovered in a volume of 100 µl using Sephadex column centrifugation.



## **Test of Probe Specificity**

### ***PCR and hybridization***

In order to test the specificity of biotinylated E1A probe, a hybridization experiment was designed in which two different PCR reactions were used to amplify adenovirus 2 E1A and human HLA-DQ $\alpha$  gene (Matsuse et al., 1992). The sizes of the amplified products from E1A and HLA-DQ $\alpha$  genes were 484 and 242 bp, respectively. Purified adenovirus 2 DNA (Gibco, BRL) (1, 10, and 1000 copies) was used as a template to amplify the E1A DNA. The PCR conditions to amplify E1A DNA were as described above (page 9). Human placental DNA (50 and 500 ng) was used to amplify the HLA-DQ $\alpha$  DNA. The PCR conditions (cycling parameters and primer sequences) to amplify HLA-DQ $\alpha$  DNA were previously described (Matsuse et al., 1992). After PCR amplification of E1A or HLA-DQ $\alpha$  DNA, 35  $\mu$ l of each 50  $\mu$ l reaction was subjected to 1 % agarose gel electrophoresis. The DNA was then transferred from the gel to a Hybond N filter (Amersham). The filter was prehybridized for 2 hours at 65° C with 6X SSC (90 mM sodium citrate, 0.9 M sodium chloride), 0.1 % Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 50  $\mu$ g/ml heparin, and 0.5 % sodium lauryl sulfate (SDS). After prehybridization, 12.5 nanogram biotinylated E1A probe which had been denatured by boiling was added to the prehybridization mixture. The filter was incubated for 16 hours at 65° C. After hybridization, the filter was washed twice for a total of 40 minutes with 2 X SSC, 0.1 % SDS at room temperature followed by two washes 20 minutes each in 0.1 % SSC, 0.1 % SDS at 65° C.

### ***Non-isotopic detection of biotinylated E1A DNA***

The labeled E1A probe hybridized to the PCR product was visualized by streptavidin-alkaline phosphatase staining of the labeled DNA. Briefly, the filter was immersed in blocking buffer [phosphate buffered saline (PBS), 0.5 % Triton X, BSA fraction V (50 mg/ml)] at room temperature for 30 minutes with shaking. The filter was then rinsed in PBS and incubated with

blocking buffer containing streptavidin-alkaline phosphatase (1  $\mu\text{g/ml}$ ) (Gibco BRL) for 30 minutes at room temperature. Filter was then rinsed in PBS, washed three times for three minutes each in PBS, 0.5 % Triton X, 3 times for three minutes in PBS and three times for three minutes in alkaline phosphate (AP) 9.6 (0.1 M Tris pH 9.6, 0.1 M NaCl, 0.1 M  $\text{MgCl}_2$ ). The filter was then placed in AP 9.6 containing 0.17 mg/ml 5-bromo-4-chloro-3-indole phosphate (BCIP), 0.33 mg/ml nitro blue tetrazolium (NBT) and 0.65% dimethyl formamide (DMF) and left in the dark for 10 minutes. To stop the color reaction, the filter was then washed for 5 minutes in PBS buffer containing 2 mM EDTA.

### **Indirect *in situ* PCR on Cytospin Preparations**

The *in situ* PCR on cytopspin preparations was carried out using a modification of the method of Bagasra et al. (1993). Briefly, slides were air dried then placed on a heating block at 100° C for 2 minutes and then in 1 % paraformaldehyde in PBS for one hour. Then the slides were washed 3 times in 3X PBS, 3 times in 1X PBS. After the cells were digested with proteinase K (50  $\mu\text{g/ml}$  in PBS) for 10 minutes at 37°C, the proteinase K was inactivated by placing the slides on the heating block at 96°C for 2 minutes. Finally, the slides were washed in distilled water. After air drying the slides, a special adhesive frame (Diamed) was placed around each cluster of cytopspin cells to allow a 50  $\mu\text{l}$  space for the PCR reaction mixture. The "hot start" technique with TaqStart antibody (Clontech, USA) directed against Taq polymerase (equal volume) was used to prevent nonspecific amplification. TaqStart antibody, a neutralizing monoclonal antibody to Taq polymerase, is used to block polymerase activity during set up of the PCR reaction at room temperature (Kellogg, et al., 1994). During the first denaturation step at 90°C or higher in thermal cycling, the activity of Taq polymerase is restored by denaturation of the thermolabile antibody. The enzyme can then begin to synthesize DNA by extension of

primers bound to the specific target sites at elevated temperatures (Kellogg, et al., 1994). The inhibition of Taq polymerase at ambient temperature and its reactivation at temperatures above 70°C enhances the specificity and sensitivity of the PCR by preventing nonspecific amplification due to mispriming or primer oligomerization (Chou, et al., 1992). A total of 50 µl of a PCR mixture containing 10 mM Tris buffer (pH 8.4), 2 mM MgCl<sub>2</sub>, 50 mM KCl, 0.01 % gelatin, 200 µM dNTP, 1.5 µM E1A primers, 2.5 units of Taq polymerase mixed with 2.5 units of TaqStart antibody was then placed on each cytospin preparation and sealed with a coverslip (Diamed). The slides were then placed on a PTC-100 thermocycler (M.J. Research) for amplification. Initially, the DNA was denatured at 94°C for 2 minutes. This was followed by an optimal number of cycles as determined below (page 20) of denaturation at 94°C for 1 minute, annealing at 63° C for 1 minute and extension at 72° C for 2 minutes. To ensure complete extension of the PCR product, the last PCR cycle was followed by 7 minutes for primer extension at 72° C. As a negative control, cytospin preparations were covered with the same PCR mixture without Taq polymerase. As a control to assess the effects of PCR cycling on subsequent *in situ* hybridization step, cytospins of adenovirus infected A549 cells were incubated with the same PCR mixture containing no primers, dNTP or Taq polymerase. After the PCR, the coverslips were removed by cutting and the PCR solution was recovered for analysis of PCR product diffusion out of the cells. The cells were then fixed in 4% paraformaldehyde (pH 7.0) for 5 minutes, washed in PBS buffer for 5 minutes, dehydrated in 70% and 95% ethanol and air dried. Visualization of intracellular PCR products was achieved indirectly by *in situ* hybridization (Hogg et al., 1989) using the biotinylated E1A probe. Briefly, cytospin preparations were covered with 20 µl of hybridization mixture and then coverslipped. The hybridization mixture contained 45 % formamide, 250 µg/ml salmon sperm DNA, 25 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 6.5), 10 % dextran sulfate and 16.6 pg/µl of biotinylated E1A probe. The DNA in both the probe and cells were then denatured

by heating the slides at 95° C for 10 minutes in a preheated water bath. The DNA was then allowed to hybridize for 18 hours at 37° C in covered plastic dish sealed in a plastic bag lined with wet towels. After hybridization, the coverslips were rinsed off in 2 X SSC buffer, and the slides were washed twice with 2 X SSC for 5 minutes, twice with 0.1 X SSC for 5 minutes, 2 X SSC for 5 minutes. They were then dehydrated twice in 70% for 10 minutes, once in 95% ethanol for 5 minutes, and air dried. An *in situ* PCR on uninfected A549 cells treated in the same manner as the cytospin preparations of Graham 293 was used, in addition to the controls listed above, as a negative control (Table 4). As a control to assess the quality of detection system, two cytospin preparations of adenovirus-infected A549 cells, which were not subjected to PCR, were also hybridized with the biotinylated E1A probe. The biotinylated pUC13 probe was used as a control to monitor non-specific binding of the biotinylated probe. To visualize the probe DNA bound to the *in situ* PCR product, non-isotopic detection of biotinylated DNA was performed as described above for its detection after Southern hybridization (pages 11 to 12). To stop the color reaction, the slides were then washed for 5 minutes in PBS buffer containing 2 mM EDTA. The slides were then washed in distilled water, air dried, coverslipped with Kaiser glycerol jelly and examined under the light microscope for nuclear staining.

### **Indirect *in situ* PCR on Paraffin Embedded Sections**

The *in situ* PCR on paraffin embedded sections was carried out using a modification of method of Nuovo et al. (1991). Briefly, sections were deparaffinized in HistoClear (Diamed), dehydrated in absolute methanol and air dried. Sections were then digested with 1 mg/ml pepsin in 0.2 N HCl for 15 minutes at room temperature, washed three times with 2 X SSC buffer followed by dehydration in 70% ethanol twice for 10 minutes and in 95% ethanol for 5 minutes. *In situ* amplification of E1A DNA was performed as described for cytospin preparations.

Cycling parameters and detection of *in situ* PCR products were also the same as those described for cytospin preparations. Controls for *in situ* amplification were equivalent as those described for cytospin preparations (pages 12, 13), except that the cells had been fixed in formalin and embedded in paraffin.

### **Optimal Conditions for Indirect *in situ* PCR**

To optimize *in situ* amplification of E1A DNA, indirect *in situ* PCR was performed on paraffin embedded sections of Graham 293 cells using different  $\text{MgCl}_2$  and primers concentrations, and cycling parameters. The optimal  $\text{MgCl}_2$  concentration was determined by subjecting paraffin embedded sections of Graham 293 cells to *in situ* PCR in 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 mM  $\text{MgCl}_2$  for 40 cycles each with the other components of the PCR reaction mixture as described above (pages 12 and 13). In further experiments, to determine the optimal primer concentrations, we tested E1A primers at 0.5, 1.0, 1.5, 2.0 and 2.5  $\mu\text{M}$  using 2 mM  $\text{MgCl}_2$  for 40 cycles on paraffin embedded sections of Graham 293 cells. Optimal  $\text{MgCl}_2$  and primer concentrations that were obtained from indirect *in situ* PCR on paraffin embedded sections of Graham 293 cells were used for cytospin preparations. PCR cycle number was varied from 20 to 40 cycles for both cytospin preparations and paraffin embedded sections of Graham 293 cells using the above optimized conditions. The other components and conditions of the PCR reaction were the same as those described above (pages 12 to 13).

### **Optimal Protease Conditions**

To optimize *in situ* DNA amplification, it is necessary to optimize protease digestion because either overdigestion or underdigestion can hinder the successful outcome of *in situ* PCR

(Komminoth and Long, 1993). With overdigestion, it is possible that large amounts of PCR products may diffuse into adjacent cells or into the solution phase of the PCR reaction during thermal cycling, and this can lead to false positive results in a mixed cell population. On the other hand, underdigestion can hinder penetration of PCR reagents and result in poor amplification. To maximize the efficiency of *in situ* E1A DNA amplification and to prevent diffusion of amplified products, cytopins and paraffin embedded sections of Graham 293 cells were subjected to indirect *situ* PCR after treatment of the preparations with different digestion and permeabilization conditions. Optimal  $\text{MgCl}_2$  (2 mM) and primer (1.5  $\mu\text{M}$ ) concentrations, and cycle number (30) that had been obtained from indirect *in situ* PCR on paraffin embedded sections of Graham 293 cells (see above) were used for the protease optimization. The other components and conditions of the PCR reactions were the same as those described above (page 12). Cytospin preparations of Graham 293 cells were permeabilized with 50  $\mu\text{g}/\text{ml}$  proteinase K at 37°C for 5, 10, 15, 30, 60, and 120 minutes. Paraffin embedded sections were digested with either 1 mg/ml pepsin in 0.2 N HCl at 37° C or room temperature for 10 or 15 minutes or with 1 to 2 mg/ml pepsin at 37° C or room temperature for 10 to 15 minutes and then subjected to indirect *in situ* PCR as described above.

To test the possibility of diffusion of amplified E1A products, after the PCR, 35  $\mu\text{l}$  of PCR solution was recovered and subjected to 1 % agarose gel electrophoresis and the intensity of E1A band was documented by ethidium bromide staining. The DNA was then transferred from the gel to a Hybond N filter. After hybridization of the DNA on the filter with a radiolabeled E1A probe, the filter was subjected to autoradiography. The radiolabeled E1A probe was made by the random priming technique as described above for the biotinylated E1A probe except that 50  $\mu\text{Ci}$  of  $\alpha\text{-}^{32}\text{P}$ -dCTP (Amersham) was used in place of Bio-11-dUTP, cold dCTP was omitted from the reaction mixture and 72  $\mu\text{M}$  dTTP was used.

## **Inhibition of PCR by Agarose**

Compared with PCR in the solution phase, *in situ* amplification of DNA in paraffin embedded sections is less efficient (Murray, 1993) and this may be partly due to Taq polymerase inhibiting impurities in the agarose gel (Feinberg and Vogelstein, 1983). To determine whether or not the agarose present in sections of paraffin embedded Graham 293 cells as a consequence of the embedding process could inhibit *in situ* E1A DNA amplification, paraffin embedded sections of these cells were covered with the E1A PCR mixture that was spiked with  $10^5$  copies of adenovirus 2 DNA and subjected to PCR. The permeabilization step and PCR conditions were the same as those described for indirect *in situ* PCR on paraffin embedded sections using optimal conditions (see above, pages 15 to 17). As controls, paraffin embedded sections of uninfected A549 cells, uninfected guinea pig lungs and a slide preparation with no section were also subjected to the same adenovirus 2 DNA-spiked PCR. After completion of the PCR reaction, 35  $\mu$ l of the PCR solution covering each of the slides was removed and subjected to 1 % gel electrophoresis. The intensity of E1A PCR product was determined by ethidium bromide staining. Furthermore, to study the nonspecific binding of the PCR products generated in the solution phase to the sections, *in situ* hybridization as described for indirect *in situ* PCR on paraffin embedded sections (see pages 12 to 15) was performed on the cell preparations after the removal of the PCR solution.

## **Indirect *in situ* PCR on Lungs and Lymph Nodes**

Using optimal conditions established for indirect *in situ* PCR on paraffin embedded sections of Graham 293 cells, indirect *in situ* PCR was performed on paraffin embedded sections of latently infected guinea pig lungs and lungs and lymph nodes from COPD and non-COPD patients. Controls for *in situ* amplification were also the same as those described for paraffin

embedded sections of Graham 293 cells (pages 15 to 16). An additional negative control for the specificity of the amplification step included the use of uninfected guinea pig lungs.

### **Direct *in situ* PCR**

Using optimal conditions established for indirect *in situ* PCR, direct *in situ* PCR was performed on cytopsin preparations of Graham 293 cells and paraffin embedded sections of Graham 293 cells, latently infected guinea pig lungs and lungs from COPD patients. The process of permeabilization and direct *in situ* PCR was as described for indirect *in situ* PCR (pages 15 to 17) with the following exceptions: in the PCR reaction mixture, 33% of dTTP was replaced by Biotin-11-dUTP. The total volume of the reaction mixture was 20  $\mu$ l. The *in situ* hybridization step utilizing the biotinylated E1A probe was omitted and instead, PCR generated products that had incorporated the biotin-labeled nucleotide were directly visualized by non-isotopic detection as described above (pages 11 to 12). The slides were then examined under the light microscope for nuclear staining. Controls for specificity of the amplification step included the use of known negative samples (uninfected A549 cells and uninfected guinea pig lungs). An additional control included the omission of E1A primers from the PCR mixture to detect nonspecific signals generated by mispriming and DNA repair. To examine the possibility of diffusion of amplified labeled product from cytopsin preparations, 15  $\mu$ l of reaction mixture was recovered and subjected to 1 % agarose gel electrophoresis and the intensity of E1A band was documented by ethidium bromide staining. The DNA was then transferred from the gel to Hybond N filters and biotin-labeled products were visualized by the streptavidin-alkaline phosphatase method (page 11 to 12).



## RESULTS

### Probe Specificity

The results obtained from the PCR and Southern hybridization experiments confirmed the expected specificity of the biotinylated E1A probe. E1A probe hybridized exclusively with 484 bp amplification product of the adenovirus 2 E1A DNA; it did not hybridize with 242 bp amplified product of the human HLA-DQ $\alpha$  gene (Fig. 1).

### Optimal PCR Conditions

Strong nuclear staining was evident when 2 mM MgCl<sub>2</sub> was used to perform *in situ* PCR on paraffin embedded sections of Graham 293 cells (Fig. 2a). A weak signal was found when the amplifying solution contained 2.5, 3.0, 3.5, 4.0 or 4.5 mM MgCl<sub>2</sub> (data not shown). No nuclear staining was observed when the MgCl<sub>2</sub> concentration was 1.5 mM. A similar study of primers concentrations on paraffin embedded sections showed that the signal was stronger with 1.5  $\mu$ M primers compared to 0.5, 1.0, 2.0 and 2.5  $\mu$ M primers (data not shown).

Nuclear staining was first evident after 20 cycles of PCR (data not shown) and was strongest after 30 cycles for both cytospin preparations and paraffin embedded sections of Graham 293 cells (Fig. 3a). After 40 cycles, a more diffuse signal was present in the nucleus and cytoplasm of the cells in paraffin embedded sections (Fig. 2a). In the case of cytospin preparations, an increase in number of cycles from 30 to 40 did not increase the signal, which remained predominantly nuclear. The optimized PCR conditions for cytospin and paraffin embedded preparations of Graham 293 cells are summarized in Table 1.

## Optimal Protease Conditions

### *Cytospin preparations*

Permeabilization of cytospin preparations of Graham 293 cells with 50 µg/ml proteinase K for 5 minutes at 37°C resulted in strong nuclear staining (Fig. 4a). The above conditions resulted in 40% cell loss (Table 2). Under these conditions, approximately 60% of the remaining cells after PCR exhibited nuclear staining (Table 4). The background cytoplasmic staining was very low, giving a strong signal to background ratio. Labeled nuclei appeared round and purple. When proteinase K digestion was extended to 10 minutes, 90 % of the cells were lost. After 15 minutes of digestion, more than 90 % of the cells were lost. Compared to 5 minutes of proteinase K digestion, there was no apparent increase in cytoplasmic staining when digestion was extended to 10 or 15 minutes (data not shown). Pretreatment with 50 µg/ml proteinase K at 37° C for 20 minutes or longer prior to indirect *in situ* PCR resulted in a complete loss of cells.

After indirect *in situ* PCR on cytospin preparations of Graham 293 cells that had been permeabilized with 50 µg/ml proteinase K at 37° C for 15 minutes or more, a strong ethidium bromide stained band corresponding to the 486 bp E1A PCR product was observed when 35 µl of reaction solution was subjected to 1% agarose gel electrophoresis (Fig.5). The intensity of the E1A band observed was the same after 15 minutes of digestion or longer. A weak E1A band was evident when Graham 293 cells were permeabilized for 10 minutes and no E1A band was found after 5 minutes digestion. These results suggest that diffusion of the amplified E1A product occurred during *in situ* PCR on cytospin preparations of Graham 293 cells. Effects of proteinase K digestion on product diffusion and the outcome of indirect *in situ* PCR on cytospin preparations of Graham 293 cells are summarized in Table 2. Digestion with proteinase K at 50 µg/ml for 5 minutes at 37°C was considered optimal (Table 1).

### ***Paraffin embedded sections***

*In situ* PCR on paraffin embedded sections of Graham 293 cells that had been digested with 1 mg/ml pepsin in 0.2 N HCl for 10 minutes at room temperature resulted in nuclear staining with minor cytoplasmic staining (Fig. 3a). Compared to cytospin preparations, nuclear staining was weaker when *in situ* PCR was performed on paraffin embedded sections of Graham 293 cells (compare Fig. 4a to 3a) with approximately 40 % of the cells showing nuclear staining (Table 4). Nuclear and cytoplasmic staining was increased when pepsin digestion was extended to 15 minutes (Table 3). On the other hand, digestion at 37°C for 10 minutes resulted in substantial background cytoplasmic staining and extensive damage to cell morphology (Fig. 6a). Digestion with 1 to 2 mg/ml pepsin in the absence of HCl for 10 to 15 minutes at room temperature or 37°C, resulted in poor nuclear staining (data not shown). Effects of pepsin digestion on the outcome of indirect *in situ* PCR on paraffin embedded sections of Graham 293 cell is summarized in Table 3. Digestion with 1 mg/ml of pepsin in 0.2 N HCl for 10 minutes at room temperature was considered optimal (Table 1).

When E1A *in situ* PCR was performed on paraffin embedded sections of Graham 293 cells that had been digested with 1 mg/ml pepsin in 0.2 N HCl at room temperature or 37°C for 10 or 15 minutes the 486 bp band corresponding to E1A PCR product was not observed on 1 % agarose gel (data not shown). However, after Southern hybridization with a radiolabeled probe the E1A band was detected on the corresponding autoradiogram (Fig. 7). The E1A band intensity was greater after 15 minutes of pepsin digestion at room temperature compared with 10 minutes digestion at the same temperature. The intensity of E1A band did not increase after digestion for 10 minutes at 37°C when compared to the same digestion for 15 minutes at room temperature (data not shown).

## **Controls**

Using these conditions of optimal permeabilization and PCR (Table 1), negative controls for *in situ* PCR on cytospin and paraffin embedded preparations of uninfected A549 cells or of Graham 293 cells where Taq polymerase was omitted from the PCR mixture showed no staining (Fig. 3b, 4b, Table 4). Even when the temperature of pepsin digestion of paraffin embedded Graham 293 cells was raised to 37°C indirect *in situ* PCR in the absence of Taq polymerase resulted in no staining (Fig. 6b). The use of irrelevant probe, biotinylated pUC13, during the *in situ* hybridization also gave negative results in paraffin embedded sections of Graham 293 cells that had been subjected to E1A amplification (data not shown). The pUC13 probe was not tested on cytospin preparations. Indirect *in situ* PCR on adenovirus infected A549 cells in the absence of E1A primers, dNTP and Taq polymerase resulted in nuclear staining in 50% of cytospin preparations and 70% of paraffin embedded sections (data not shown). The number of positive cells was slightly higher than that observed by standard *in situ* hybridization with the same probe alone.

Agarose gel analysis of possible PCR products that had diffused into the PCR solution showed that after cytospin preparations of uninfected A549 cells were digested with 50 µg/ ml of proteinase K at 37°C for 5 minutes indirect *in situ* PCR on these cells did not give a band (Fig. 5, lane g). An equivalent negative control for paraffin embedded cells, uninfected A549 cells digested with 1mg/ml pepsin in 0.2 N HCl for 10 or 15 minutes at room temperature, also showed no E1A band (Fig. 7, lanes g and k).

## **Inhibition of PCR by Agarose**

In experiments to determine whether agarose present in sections of paraffin embedded cells inhibits *in situ* E1A amplification, 10<sup>5</sup> copies of adenovirus 2 DNA were added to the PCR

reaction used for amplification. Agarose gel electrophoresis showed a band corresponding to the expected E1A PCR product in the solution recovered from the paraffin embedded sections of Graham 293 cells, uninfected A549 cells and uninfected guinea pig lungs (Fig. 8, lanes, 2, 3, and 4 respectively). The above E1A bands were similar in intensity. Compared to the control slide without a paraffin section, the E1A band observed from paraffin embedded sections was of weaker intensity. However, *in situ* hybridization with biotinylated E1A probe of paraffin embedded sections of Graham 293 cells (Fig. 9a), uninfected guinea pig lungs (Fig. 9c) and uninfected A549 cells (data not shown) that had been subjected to adenovirus spiked amplification resulted in nuclear and cytoplasmic staining. Since no staining was observed on the adjacent sections where Taq polymerase was omitted from the PCR mixture (Fig. 9b, 9d), the staining observed on the uninfected guinea pig lung and the A549 cells most likely represents binding of the amplified product of the spiked adenovirus 2 DNA. In the case of the Graham 293 cells, it could be the result of a combination of these false positive staining and genuine amplification in the nuclei of these cells.

### **Indirect *in situ* PCR on Lungs and Lymph Nodes**

*In situ* PCR on paraffin embedded sections of latently infected guinea pig lungs revealed nuclear staining in alveolar and bronchiolar epithelial cells (Fig. 10a, 10c). Examination at higher power to identify E1A positive alveolar epithelial cells type showed that type II pneumocytes were the most common positive cell. Of the 10 paraffin embedded sections of lung from the two latently infected guinea pigs, only 4 sections (2 sections per animal) showed evidence of E1A localization in epithelial cells (Table 4). In addition, only one or two cells with nuclear staining were observed on each section. Moreover, nuclear staining in a bronchiolar epithelial cell was observed on only one section from one animal. No other staining was

observed on this section. No staining was seen on an adjacent section where Taq polymerase was omitted from the PCR mixture (Fig. 10b, 10d, Table 4). The use of irrelevant probe, pUC 13, on adjacent sections after *in situ* amplification did not show any staining (data not shown). Indirect *in situ* PCR on uninfected lungs did not show any staining (Table 4).

Application of indirect *in situ* PCR to six paraffin embedded sections of lungs from COPD patients (2 slides per patient) showed E1A localization in only one of the two sections from two patients (Fig. 11a). Again only one or two E1A positive cells were found on each section. Examination of these sections at higher magnification showed that E1A DNA was localized to alveolar epithelial cells. No nuclear staining was observed on an adjacent section where Taq polymerase was omitted from the PCR mixture (Fig. 11b). The hybridization of irrelevant probe, pUC13, on the adjacent section after *in situ* amplification did not result in any staining (data not shown). No nuclear staining was observed when indirect *in situ* PCR was performed on six paraffin embedded sections (2 sections per patient) of lymph nodes from COPD patients (data not shown). Large amounts of carbon and other unidentified brown materials, which most likely are due to air pollution, were present on these sections of lymph node (Table 5).

Indirect *in situ* PCR on two E1A positive blocks of lymph nodes from non-COPD patients (2 slides per block) showed evidence of E1A DNA localization in all 4 sections examined. Between 9 to 40 cells with nuclear staining were found on these sections (Tables 4 and 5). Examination of these sections at higher magnification showed that the majority of the nuclear staining was restricted to the follicular mantle and the capsule of the lymph nodes (Fig. 12c, 12d). There was no detectable viral E1A DNA in individual cells in the germinal centers, but E1A DNA was detected in 2 isolated cells around the germinal centers in these sections (Fig. 12a). All these positive cells appear to be larger than lymphocytes. Nuclear staining was more

frequent in a lymph node that had lower amounts of carbon and other contaminants from air pollution (Table 5, block B). Nuclear staining was less frequent on the adjacent sections where Taq polymerase was omitted from the PCR mixture where between 3 to 21 positive cells were present (Table 4 and 5), and again these were primarily in the follicular mantle and capsule areas with no nuclear staining in or around the germinal centers. Standard *in situ* hybridization with biotinylated E1A probe showed evidence of nuclear staining in capsule and follicular mantle areas, but there was no detectable E1A DNA staining in or around the germinal centers. Compared to indirect *in situ* PCR in the absence of Taq polymerase, standard *in situ* hybridization showed less frequent nuclear staining (Table 5). The use of irrelevant probe, biotinylated pUC13, on adjacent sections did not result in any staining (data not shown). No nuclear staining was observed when indirect *in situ* PCR was performed on two sections from an E1A negative block of lymph nodes from a non-COPD patient (Tables 4 and 5). It should be noted that these two sections, like those of the lymph nodes from COPD patients, which proved to be negative by indirect *in situ* PCR, also contained high amounts of carbon and other contaminants. Standard *in situ* hybridization on these sections did not result in any staining (Table 5).

### **Direct *in situ* PCR**

Using optimal conditions established for indirect *in situ* PCR, direct *in situ* PCR on cytospin preparations of Graham 293 cells yielded strong nuclear signals in approximately 70% of the cells with either no cytoplasmic staining or faint staining (Table 6, Fig. 13a). Labeled nuclei appear round and dark purple. Ethidium bromide staining did not allow detection of an amplified E1A band when solutions recovered after the PCR on cytospins preparations of Graham 293 cells were subjected to 1% agarose gel electrophoresis, but the E1A band was detected after the colorimetric staining of the Hybond N filter after Southern transfer of the DNA

on this gel (Fig. 14). Direct *in situ* PCR on paraffin embedded sections of Graham 293 cells resulted in nuclear staining in approximately 50% of the cells (Table 6, Fig. 15a). Generally, the intensity of cytoplasmic staining on these paraffin embedded sections was lower compared to those subjected to indirect *in situ* PCR.

Omission of primers from the amplifying solution resulted in the detection of false positive nuclear staining in both cytopins and paraffin embedded sections of Graham 293 cells (Table 6, Fig. 13b, 15b). These false positive signals were evident in approximately 20% of cells of cytospin preparations of Graham 293, whereas only about 1% of cells in paraffin embedded sections of Graham 293 showed false positive nuclear staining. Nonspecific signals were also detected in 20% of control experiments using direct *in situ* PCR on cytospin preparations of uninfected A549 cells (Table 6, Fig. 16a). Nuclear staining was less frequent when E1A primers were omitted from the amplifying solution (Fig. 16b). No nuclear staining was observed when direct *in situ* PCR was performed on paraffin embedded sections of uninfected A549 cells (Table 6). Direct *in situ* PCR on cytospin and paraffin embedded sections of adenovirus infected A549 cells showed diffuse nuclear and cytoplasmic staining in 100% of the cells (Fig. 17a). When E1A primers were omitted from the PCR mixture, nonspecific false positive signal was observed in 50% of both infected A549 cell preparations (Table 6, Fig. 17b). When E1A primers were included in the PCR mixture, a strong ethidium bromide stained E1A band was observed when the supernatant from these infected cells was subjected to 1% agarose gel electrophoresis (data not shown). That this band represented E1A DNA was shown after non-isotopic detection of the biotinylated DNA on the corresponding Hybond N filter (Fig. 14).

Direct *in situ* PCR on one section of paraffin embedded lung from a latently infected guinea pig revealed 4 alveolar epithelial cells with nuclear staining (Table 6). Figure 18a illustrates one of these positive cells. This cellular localization of E1A DNA was similar to that



found by indirect *in situ* PCR. No nuclear staining was observed on the adjacent section where the primers had been omitted from the PCR mixture (Fig. 18b), or in lung sections from uninfected guinea pigs (Table 6).

Application of direct *in situ* PCR on one section of paraffin embedded human lung revealed one alveolar epithelial cell with nuclear staining (Table 6, Fig. 19a). No staining was seen on an adjacent section where primers were omitted from the PCR reaction (Fig. 19b).

## Discussion

The purpose of this study was to develop a protocol capable of detecting low copy numbers of adenovirus E1A DNA *in situ* in histological preparations of human lungs and lymph nodes. Graham 293 cells and latently infected guinea pigs were used as model systems to develop this procedure. The results show that adenovirus E1A DNA can be demonstrated *in situ* in the nuclei of Graham 293 cells by indirect *in situ* PCR both on cytospin and paraffin embedded specimens. These experiments were used to characterize the conditions that optimize the *in situ* detection of PCR-amplified E1A DNA in Graham 293 cells. Our results also support the findings of others (Long, et al., 1992; Sallstrom, et al., 1993) claiming that indirect *in situ* PCR which uses the *in situ* hybridization step for added specificity in the detection of amplified E1A DNA is superior to direct *in situ* PCR. Our results showed that application of direct *in situ* PCR on cytospin preparations of Graham 293 and A549 cells yielded a significant number of false positive results. However, compared to cytospin preparations, direct *in situ* PCR on paraffin embedded sections of Graham 293 and A549 cells showed significantly lower or absent false positive signals, respectively; the reason for that will be discussed in detail below. Application of indirect *in situ* PCR on guinea pig lungs that had been latently infected with adenovirus 5 revealed nuclear staining in the epithelia of bronchiole and alveolar walls. Nuclear

staining was also observed in alveolar epithelial cells when indirect *in situ* PCR was performed on paraffin embedded sections of human lungs.

The protocols used in our studies were based on those of Bagasra et. al. (1993) and Nuovo (1991) but were modified to maximize nuclear staining in cytospin preparations and paraffin embedded sections of Graham 293 cells. As the success of indirect *in situ* PCR on Graham 293 cells depends largely on the way these cells have been digested before amplification, protease pretreatment of both cytospin and paraffin embedded preparations were analyzed in detail. In this study, successful *in situ* amplification and localization of adenovirus E1A DNA was achieved when cytospin preparations and paraffin embedded sections of these cells had been digested with 50 µg/ml proteinase K for 5 minutes at 37°C and with 1 mg/ml pepsin in 0.2 N HCl for 10 minutes at room temperature, respectively. Inadequate digestion of paraffin embedded sections of Graham 293 cells in the absence of HCl, even when the temperature was elevated to 37°C, resulted in poor nuclear staining. The primary reason for protease digestion is to facilitate entry of PCR reagents into the cell. Therefore, insufficient protease treatment limits the accessibility of nuclear DNA to PCR reagents. In addition, protease removes DNA-histone cross linking, which occurs as a result of formalin fixation (Nuovo, et al., 1991). Such cross linking of histone protein to DNA is likely to prevent the progression of Taq polymerase along the native DNA template (Junqueira, et al., 1977). With insufficient digestion, a large numbers of these cross links may persist and decrease the efficiency of amplification thus lead to poor nuclear staining.

Excessive protease treatment, on the other hand, results in diffusion of amplified E1A products out of the nucleus and consequently poor nuclear staining, damage to cell morphology, and loss of cells or sections from the slide. Diffusion artifacts represented the most significant problem of indirect *in situ* PCR on paraffin embedded sections of Graham 293 cells. Diffusion of

amplified E1A products produced a significant cytoplasmic staining in paraffin embedded sections of Graham 293 cells that had been subjected to more extensive protease treatment. It is possible that excessive digestion may result in total removal of nuclear protein cross links secondary to formalin fixation and this would allow part of amplified E1A products to leak out of the nucleus and cell into the PCR solution. It is hypothesized that PCR products, which have diffused out of the cell, may serve as templates for extracellular amplification, a process that is probably far more efficient than intracellular amplification. When comparing the relative efficiency of extracellular and intracellular amplification after 40 cycles, Ray and coworkers (1995) showed that the band intensity was greater in the supernatant fraction composed of extracellular amplified product consistent with the notion of greater efficiency of PCR on liberated DNA compared with intracellular amplification. These extracellularly amplified DNAs have the potential to adhere to exposed cellular basic proteins resulting in cytoplasmic staining.

In contrast to paraffin embedded sections, background cytoplasmic staining was not a problem after indirect *in situ* PCR in cytopsin preparations. One possible explanation for this difference is that in cytopsin the almost intact cell membrane may hinder extracellularly amplified E1A products to diffuse back into the cell, whereas after sectioning paraffin embedded cells, most of the cells are left without intact cell membrane and extracellularly generated E1A products can freely bind to cytoplasmic proteins. Results from other studies (Long, et al., 1993; Komminoth, et al., 1992) on nonspecific cellular binding and uptake of extracellularly generated PCR products by cell suspensions support the view that intact cell membrane can prevent back diffusion of large amplified products. Long and coworkers (1993) placed uninfected fixed human fibroblasts into a PCR tube in which viral DNA sequences of different lengths were being tested by amplification extracellularly. After the PCR, the cells were washed and subjected to *in situ* hybridization using specific oligonucleotide probes. The results indicate that false positive

signals were far higher when smaller PCR products less than 150 bp were generated possibly due to back diffusion of the extracellular PCR products into the fixed fibroblasts during thermal cycling. In addition, diffusion artifacts were significantly reduced when biotinylated nucleotides instead of unmodified ones were used to generate bulkier and therefore less diffusible amplified products (Komminoth, et al., 1992). Moreover, our findings of positive nuclear and cytoplasmic staining after using a PCR reaction spiked with adenovirus DNA during indirect *in situ* PCR on paraffin embedded sections of Graham 293 cells (Fig. 7a), uninfected A549 cells and uninfected guinea pig lungs (Fig. 7c) support the view that nonspecific cytoplasmic staining in paraffin embedded sections is mainly due to binding of extracellularly produced PCR products onto the sections. Furthermore, the fact that on agarose gel electrophoresis the band intensity of E1A products in the PCR solution recovered from these sections was weaker when compared to controls with no sections (Fig. 8) suggests that some of amplified E1A products must have adhered to the sections. Another explanation is the possibility that in paraffin embedded sections PCR amplification in fixed tissue is less efficient. Adenovirus templates may bind to proteins and becomes less available for amplification leading to reduced amplification efficiency. Therefore, the implication of these results is that in order to minimize cytoplasmic staining in paraffin embedded sections of Graham 293 cells, outward diffusion of amplified E1A product had to be reduced. One approach was to optimize protease digestion in such a way that it would permit penetration of PCR reagents into the nucleus and free DNA from histone to allow successful *in situ* amplification of target DNA while avoiding outward diffusion of amplified products. Optimal localization of amplified E1A DNA was found when paraffin embedded sections of Graham 293 cells were digested with 1 mg/ml pepsin in 0.2 N HCl for 10 min at room temperature.

Another approach taken to minimize outward diffusion of amplified product suggested by

studies by Komminoth and coworkers (1992) was the use of a relatively low numbers of PCR cycles. When the numbers of PCR cycles was reduced from 40 to 30, there was a significant reduction in cytoplasmic staining on paraffin embedded sections of Graham 293 cells. However, PCR cycle numbers lower than 20 also resulted in poor nuclear staining in both cytospin and paraffin embedded sections of Graham 293 cells. This is consistent with the findings of Haase et. al. (1990) that *in situ* amplification is less efficient than solution phase PCR and therefore more cycles may be required to achieve the same degree of amplification.

Successful *in situ* amplification of E1A DNA in paraffin embedded preparations of Graham 293 cells was achieved only when 2 mM  $MgCl_2$  was used. The use of either lower (1.5 mM) or higher concentrations of  $MgCl_2$  (higher than 2 mM) did not result in nuclear staining. This is contrary to other studies which report the use of much higher concentrations of  $MgCl_2$  (4.0 to 4.5 mM) for successful *in situ* amplification (Nuovo et al., 1991; Bagasra et al., 1993). Low concentrations of  $MgCl_2$  can affect the yield of amplified product. If  $MgCl_2$  concentration is very low, the extension reaction is impaired as magnesium ions are required as a cofactor for the DNA polymerase activity. Most investigators in this field (Nuovo et al., 1991; Bagasra et al., 1993) believe that it is the sequestration of  $MgCl_2$  ion on cellular proteins and the glass slide that increases the need for higher concentrations of  $MgCl_2$  during *in situ* PCR. In our study, sequestration of  $MgCl_2$  did not appear to be a significant problem since 2.0 mM  $MgCl_2$  resulted in successful *in situ* amplification of E1A DNA in Graham 293 cells. It is not clear why higher concentrations of  $MgCl_2$ , which have been reported by others to allow amplification (Nuovo, et al., 1991; Bagasras, et al., 1993), did not result in nuclear staining in our study. In solution phase PCR, a high concentration of  $MgCl_2$  results in the accumulation of non-specific amplification products (Saiki, 1989). Recently, Martinez et al. (1995) showed that the use of high  $MgCl_2$  concentration resulted in nonspecific nuclear staining by direct *in situ* PCR. Our use of a

biotinylated E1A DNA specific probe would not allow the detection of any nonspecific amplification products and, therefore, it is conceivable that at higher  $\text{MgCl}_2$  concentrations nonspecific amplification took place but was not detected in our case.

The success of indirect *in situ* PCR on Graham 293 cells also depends on optimal concentrations of E1A primers. Strong nuclear staining was observed when E1A primer concentrations were 1.5  $\mu\text{M}$ . This is three times higher than the concentration used for solution phase PCR. This relatively high concentration of primers probably reflects their partial sequestration on the glass slide and by cellular proteins. However, the use of primer concentrations higher than 1.5  $\mu\text{M}$  is of particular interest since it resulted in weak nuclear staining. Target specific amplification during PCR suffers from several side reactions that include mispriming and primer oligomerization. One interpretation of our result is that at particularly high concentrations of primers, target specific amplification during PCR may be inhibited by mispriming or primer oligomerization. Another interesting modification that has been used to enhance the *in situ* PCR is the use of multiple primer pairs (Haase, et al., 1990) which are designed to amplify large PCR products. As the final PCR product is longer, it is affected less by diffusion and thus increases the power of its subsequent detection. However, the primer multiplicity most likely increases the probability of mispriming and primer oligomerization leading to increased nonspecific amplification (Nuovo, et al., 1991). In addition, Ray et. al. (1994) showed that the length of DNA fragment to be amplified did not affect the final detection of the amplified DNA after *in situ* hybridization. At the same time Nuovo and coworkers (1991) showed that the one copy of HPV 16 DNA in the cervical carcinoma cell line (SiHa) was detectable by indirect *in situ* PCR with a single primer pair that had a target sequence of 450 bp only if "hot start" modification was employed. We also achieved excellent localization of the amplified E1A product using a single primer pair to amplify a 486 bp target in conjunction

with “hot start” PCR.

The effect of PCR inhibitors was also considered in this study by checking the suitability of paraffin embedded sections of cultured cells for PCR amplification. The most likely candidate as a cause of inhibition of PCR in these sections was the agarose that had been mixed with the cells before embedding in paraffin. Commercially available agarose are sometimes contaminated with poorly characterized polysaccharides which are potent inhibitors of many of the enzymes commonly used in molecular cloning including Taq polymerase (Sambrook, et al., 1989). In our study, we used special grade low melting agarose that was screened by the manufacturer for the presence of enzyme inhibitors. Although use of low melting agarose has reduced the problem of contamination, there are occasions when enzyme inhibition is seen. Feinberg and Coworkers (1983) showed that contaminants from agarose gel sometimes inhibit nick translation. Our result confirms the findings of Chiu and coworkers (1992) that low melting agarose does not inhibit PCR amplification. However, our results demonstrate that the efficiency of indirect *in situ* amplification and localization of E1A DNA in paraffin embedded sections of Graham 293 cells was lower when compared to cytospin preparations. Approximately 40% of the Graham 293 cells of paraffin embedded sections showed evidence of E1A localization when compared to 60% of the cells on cytospin preparations. This is in agreement with other studies in which the efficiency of *in situ* amplification in paraffin embedded sections was estimated to be significantly lower than that of cytospin preparations (Komminoth, et al., 1992). This lower efficiency may be related to other differences between these two preparations and not necessarily to agarose effects. Reduced amplification efficiency on paraffin embedded sections may relate to DNA damage and loss that occur after embedding and sectioning process. Alternatively, it is possible that reduced retention of amplified products in paraffin embedded preparations due to sectioning through the cell may result in poor signal localization.

The findings from studies conducted here support the view that indirect *in situ* PCR is a technique that allows very specific detection of target sequences. There was no positive signal in either cytospin preparations or paraffin embedded sections of Graham 293 cells when Taq polymerase was excluded from PCR mixture or when the cells were subjected to *in situ* hybridization after *in situ* amplification with an irrelevant probe which in our case was biotinylated pUC13 DNA. Also no positive signal was observed when indirect *in situ* PCR was performed on uninfected A549 cells. This control is particularly important, since nonspecific amplification of DNA can occur in the presence of Taq polymerase. However, because our E1A probe hybridizes exclusively with 484 bp amplified product of adenovirus E1A DNA, nonspecific amplification, if it took place, did not result in any staining in uninfected A549 cells and consequently should not compromise the results obtained from Graham 293 cells or lung tissue.

In contrast to indirect *in situ* PCR, nonspecific nuclear staining was frequent and of strong intensity when direct *in situ* PCR was performed on cytospin preparations of Graham 293 cells when primers were omitted from the PCR reaction or preparations of uninfected A549 cells where no target DNA is present. One possible explanation for these findings include labeled nucleotide incorporation during DNA repair that occurs as a result of polymerase activity at sites where formaldehyde fixation has caused single stranded nicks in the DNA (Demkovicz-Dobrzanski and Castong, 1992). Long et. al. (1992) showed that nonspecific incorporation of labeled nucleotides into damaged DNA undergoing repair by DNA Taq polymerase may occur leading to false positive results. In addition, direct *in situ* PCR on cytospin preparations of adenovirus infected A549 cells in the absence of primers showed more frequent false positive signals when compared to that of Graham 293 or uninfected A549 cells. One possible explanation for this difference is that virus infected cells induce apoptosis (programmed cell



death) through mobilization of apoptosis specific nucleases, fragmenting and degrading DNA of host cells (Sallstrom, et al., 1993). Thus it is possible that the high degree of DNA degradation in adenovirus infected A549 cells may result in an increased DNA repair leading to increased nonspecific signals when compared to Graham 293 or uninfected A549 cells. Many creative approaches have been employed by others to minimize the DNA repair pathway. For example, others have shown that these artifacts can be somewhat reduced by repairing DNA nicks by treatment with T4 ligase before PCR (Koch, et al., 1991), or initial thermal cycling using unlabeled nucleotides (Morital, et al., 1994). However, it has not yet proven possible to eliminate completely these nonspecific pathways which interfere with accurate detection of specific signals. A possible explanation for nonspecific signals observed after direct *in situ* PCR, even after preliminary treatment to repair nicks in DNA, is mispriming. Mispriming can result in the incorporation of labeled nucleotides into nonspecific amplified products leading to false positive results. Our preliminary results showed that omitting E1A primers from the PCR mixture during direct *in situ* PCR on cytospin preparations of uninfected A549 cells resulted in reduction of nonspecific signals. This finding suggests that mispriming is the other probable cause of false positive signals observed in cytospin preparations. Hot Start modification has been used by others to overcome mispriming during direct *in situ* PCR, but this has not proven to be completely reliable. For instance, while some observers have reported that Hot Start modification of PCR has the potential to reduce mispriming (Erlich, et al., 1991; Nuovo, et al., 1993), we and others (Zehbe, et al., 1992; Komminoth and Long, 1993) have failed to eliminate nonspecific signals by such attempt. The use of labeled primers in place of labeled nucleotides during direct *in situ* PCR may have the potential to eliminate nonspecific nuclear staining due to DNA repair pathway, but mispriming could still persist. In this approach, the use of unrelated labeled primers on adjacent sections would allow us to estimate the degree of nonspecific

amplification. Therefore, although in this approach nonspecific signals due to mispriming cannot be eliminated, the frequency of such signals can be monitored using irrelevant labeled primers on adjacent sections.

Our preliminary results showed that compared to cytospin preparations, direct *in situ* PCR on paraffin embedded sections of Graham 293 cells resulted in significantly lower numbers of false positive signals. This is also in contrast to most reports (Sallstrom, et al., 1994; Zehbe, et al., 1994) that showed more frequent false positive signals in paraffin embedded tissue sections when compared to cytospin preparations. One possible explanation for the difference observed between our report and others includes the type of starting materials in paraffin embedded sections. Compared to our model system developed with live cells in culture which allows for precise control of both the duration and nature of fixation used, most of the tissue samples reported by others include archival paraffin embedded samples that had been subjected to prolonged fixation of uncertain duration. This can have adverse effects on the quality of DNA being amplified. This may also explain the higher frequency of false positive signals in tissue sections (Long et al., 1992), which were likely to have had more DNA damage due to fixation than the cultured cells. Another possible explanation for our finding is that the frequency of nonspecific signal may depend on differences in the efficiency of *in situ* amplification in paraffin embedded sections compared to cytospin preparations. In agreement with the results of indirect *in situ* PCR, our preliminary results of direct *in situ* PCR showed lower *in situ* E1A amplification signals in paraffin embedded sections of Graham 293 cells when compared to cytospin preparations. Although this suggests reduced amplification efficiency of specific products on paraffin embedded sections, results using direct *in situ* PCR on cytospin preparations of uninfected A549 cell also indicate that the frequency of nonspecific signals is amplification dependent. The implication of these results is that reduced efficiency of amplification in general

may contribute to reduced nonspecific signal in paraffin embedded sections. Moreover, direct *in situ* PCR, in the presence or absence of E1A primers, on paraffin embedded sections of uninfected A549 cells did not result in any staining. These findings suggest that mispriming and DNA repair mechanism are less important sources of error in paraffin embedded sections, even though they appear to be the two major contributing factors for nonspecific signals in cytospin preparations. Factors that contribute to low level artifacts in paraffin embedded sections of Graham 293 cells in the absence of primers are not known and require further investigation. However, lack of involvement of mispriming and DNA repair mechanism may partially explain why fewer false positive signals were observed on paraffin embedded sections of Graham 293 cells compared to cytospin preparations.

We have successfully transferred the protocol developed on Graham 293 cells to histological sections of lung tissues where our results using indirect *in situ* PCR on paraffin embedded sections show that the primary target cell was the type II alveolar epithelial cell both in latently infected guinea pig and human lungs. The epithelial cells lining the bronchiole were less frequently positive in guinea pig lungs. The absence of nuclear staining without Taq polymerase demonstrates the specificity of our indirect *in situ* PCR protocol, and confirms the relative insensitivity of standard *in situ* hybridization. The infrequent detection of adenovirus E1A DNA by standard *in situ* hybridization in COPD lungs is in agreement with previous studies (Matsuse, et al., 1992) in which adenovirus was demonstrated in only two of 60 blocks of lung tissue from these patients. In contrast, after PCR amplification, two of the six slides prepared from three blocks of lungs from two COPD patients showed evidence of E1A DNA. Similarly, of the 10 paraffin embedded sections of latently infected guinea pig lungs examined, 4 sections (2 sections per animal) showed evidence of E1A localization in epithelial cells. Therefore, our study confirms that indirect *in situ* PCR on paraffin embedded sections of lungs from COPD patients

and latently infected guinea pigs improves the detection rate when E1A DNA is present in low copy numbers.

In the present study, E1A DNA was not detected on paraffin embedded sections of lymph nodes from COPD patients by indirect *in situ* PCR. The absence of adenovirus E1A DNA in lymph nodes from COPD patients cannot completely exclude the possibility of latent adenovirus infection in these tissues. This assumption is based on two previous studies in which group C adenovirus DNA was demonstrated in peripheral blood lymphocytes and tonsils from asymptomatic adults by Southern hybridization (Green, et al., 1979; Horvath, et al., 1986). The Southern hybridization experiments used human tonsils and peripheral blood lymphocytes from asymptomatic adults and showed that between 20 to 100 copies of complete adenovirus genome were present in each cell. In contrast, lymph nodes from non-COPD patients showed E1A DNA localized to non-lymphocytic cells in the capsule and follicular mantle by both indirect *in situ* PCR and by standard *in situ* hybridization. The large amounts of carbon present in the lymph nodes in COPD patients could account for the absence of nuclear staining because these and other contaminants from air pollution may have inhibited *in situ* amplification of E1A DNA. It is also possible that the same materials can absorb the color reagents (BCIP and NBT) and therefore interfere with the color reaction used to detect the biotinylated probe. This is also consistent with the results of indirect *in situ* PCR and standard *in situ* hybridization on lymph nodes from one non-COPD patient in which nuclear staining was absent on sections with deposits from environmental pollution. Alternatively, it is possible that E1A DNA was not present in the limited number of lymph nodes from COPD patients that were so far examined.

Comparison of cellular localization of adenovirus E1A DNA by indirect *in situ* PCR in human and latently infected guinea pig lungs shows the following similarities. First, the primary target tissue in both cases was alveolar epithelium. Second, this cellular localization was also

similar to that of our previous studies (Elliot, et al., 1995; Vitalis, et al., 1996) in which E1A protein was detected in the alveolar epithelial cells of human and latently infected guinea pig lungs. Third, the use of E1A DNA localization in human and guinea pig lungs as a measure of number of E1A containing cells has yielded results which suggest that latency is typically established in only a minute fraction of the alveolar epithelial cells, from one to two E1A positive cells per paraffin embedded section. However, the use of nuclear staining detected by indirect *in situ* PCR as a measure of the number of E1A containing cells may underestimate the number of E1A positive cells in these preparations. This underestimation is reflected in the detection of E1A DNA in Graham 293 cells where every cell carries 4 to 5 copies of the target DNA. In cytopsin preparations only 60% of the cells were positive and this fell even lower in paraffin embedded preparations, where there were only 40% positive cells. This underestimation may be, in part, related to limitation of assay sensitivity. The indirect *in situ* PCR was optimized to localize E1A DNA in cytopsin preparations and paraffin embedded sections of Graham 293 cells which are known to have four to five copies of adenovirus E1A DNA. It is not known how many copies of E1A DNA are present in each latently infected cell of human and guinea pig lungs. In this regard, further optimization of *in situ* E1A DNA amplification on tissue sections may be required. Underestimation, in the case of paraffin embedded sections, may also be related to the absence of E1A DNA in some cells since their nuclei are often bisected by the sectioning. It is also possible that E1A DNA is not evenly distributed within the lung, and detection of E1A positive cell in lung sample reflects this non-random distribution. This is consistent with our previous studies (Matsuse, et al., 1992) in which repeated solution phase PCR on DNA extracted from serial sections of the same blocks of COPD and non-COPD lungs confirmed positivity for E1A DNA on 13 of 16 and 8 of 16 DNA samples, respectively.

Results of direct *in situ* PCR on paraffin embedded sections of human and latently

infected guinea pig lungs also support the localization of E1A DNA in alveolar epithelial cells. Also, although the number of specimens analyzed was limited to allow statistical comparisons, the level of detection of E1A DNA did not appear to be greater by this method than that found by the indirect method. However, because our preliminary results indicate false positive signals associated with direct *in situ* PCR on paraffin embedded sections of Graham 293 cells, complete reliance of the direct *in situ* PCR approach for the cellular localization of E1A DNA in the case of human and latently infected guinea pig lungs should be regarded with caution.

The theory regarding the progression of COPD suggests that neutrophils are important cellular effectors of inflammation (Blue and Janoff, 1978). Neutrophils can cause tissue damage through release of degradative enzymes and oxygen radicals (Weis, S.J., 1984). Recently, Coxson et. al. (in press) showed that there was a significant increase in the number of neutrophils and alveolar macrophages in lungs from COPD patients when compared to the control group with matched smoking history. Similarly, Thompson et. al. (1989) showed increased number of neutrophils in lavage fluid from patients with COPD. However, the mechanism by which neutrophils and monocytes are recruited to the lungs in COPD patients is not clear. We postulate that the presence of E1A DNA and its expression in alveolar epithelial cells may be the driving factor for enhanced recruitment of neutrophils and alveolar macrophages to the lung after exposure to cigarette smoke. *In vitro* studies showed that expression of E1A DNA in pulmonary epithelial cells can enhance the induction of proinflammatory mediators such as IL-8 and ICAM-1 (Keicho, et al., 1997, 1997). IL-8 is a potent chemoattractant and activator of neutrophils (Oppenheim, et al., 1991), whereas ICAM-1 serves as a ligand for adhesion receptor on neutrophils (Diamond, et al., 1991). Likewise, *in vivo* studies suggest that IL-8 and ICAM-1 expression was significantly higher in COPD lungs compared to control groups (Keating et al., 1994; Di Stefano, et al., 1996). Taken together with our observations, these results suggest that

after exposure to cigarette smoke, expression of E1A DNA in epithelial cells could augment the induction of IL-8 and ICAM-1 leading to an excessive PMN response to cigarette smoke inhalation. This increased recruitment of neutrophils into the lung tissue could result in irreversible lung damage.

In summary, our data showed that E1A DNA can be detected in cytospin preparations and paraffin embedded sections of Graham 293 cells using indirect *in situ* PCR technique. The use of cytospin preparations appeared to provide maximal amplification and detection of E1A DNA in these cells. In this study, we also examined the importance of various conditions such as protease pretreatment,  $MgCl_2$  and primers concentrations, and the number of PCR cycles that would enhance *in situ* amplification and localization of E1A DNA in Graham 293 cells. In our experience, the most crucial step in obtaining good amplification and localization of E1A DNA was the protease pretreatment. We postulate that protease treatment has a direct effect on amplification efficiency and retention of amplified product. Our experiments revealed significant problems of direct *in situ* PCR concerning nonspecific positive signals. Based on these findings, we conclude that direct *in situ* PCR must await for further resolution of its current limitations before it can be used as a reliable technique for *in situ* amplification. The results obtained using indirect *in situ* PCR on paraffin embedded sections of human and latently infected guinea pig lungs showed evidence of E1A DNA localization in alveolar epithelial cells. This cellular localization is similar to that found in previous studies (Elliot, et al., 1995; Vitalis, et al., 1996) by immunohistochemistry and is consistent with the conclusion reached by Matsuse and coworkers (1992) using *in situ* hybridization. Localization of adenovirus E1A DNA in alveolar epithelial cells has important implications to the pathogenesis of emphysema because the upregulation of proinflammatory agents in these cells could mediate neutrophil migration into the alveolar walls. This amplification of the inflammatory process by latent adenovirus infection

could provide a partial explanation for the fact that only a small percentage of heavy smokers develop airway obstruction.



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**Table 1. Optimal permeabilization and PCR conditions for *in situ* PCR on Graham 293 cells.**

<b>Conditions</b>	<b>Cytospins</b>	<b>Paraffin embedded cells</b>
<b>Proteinase K</b>	50 µg/ ml, 37°C, 5 min	n.d.
<b>Pepsin</b>	n.d.	1 mg/ ml, 0.2 N HCl, R.T., 10 min
<b>MgCl<sub>2</sub></b>	2.0 mM	2.0 mM
<b>E1A Primers</b>	1.5 µM	1.5 µM
<b>Cycle numbers</b>	30	30

**(n.d.)** not done

**(R.T.)** room temperature

**Table 2. Effect of proteinase K permeabilization time on outcome of indirect *in situ* PCR on cytospin preparations of Graham 293 cells**

<b>Time (min)</b>	<b>*Overall nuclear staining</b>	<b>% cell loss</b>	<b>Diffusion of amplified products</b>
<b>5</b>	+++++	40	none
<b>10</b>	++	90	minor
<b>15</b>	+	>90	significant
<b>20</b>	-	100	significant
<b>30</b>	-	100	significant
<b>60</b>	-	100	significant
<b>120</b>	-	100	significant

(\*) To calculate this, intensity of nuclear staining, % cell loss and amount of diffusion of amplified products were taken into consideration.

**Table 3. Effect of pepsin digestion conditions on outcome of indirect *in situ* PCR on paraffin embedded sections o Graham 293 cells**

Digestion Time (min)	2 mg/ ml pepsin		1 mg/ ml pepsin, 0.2 N HCl	
	R.T.	37°C	R.T.	37°C
<b>10</b>	negative	negative	++	<u>±</u>
<b>15</b>	negative	negative	+	n.d.

**(R.T.)** room temperature

**(negative)** no staining

**(++)** good nuclear staining with minor cytoplasmic background

**(+)** nuclear staining with increased cytoplasmic background

**(±)** nuclear staining with strong cytoplasmic background and damage to cell morphology

**(n.d.)** not done



**Table 4. Summary of the detection of amplified E1A DNA by indirect *in situ* PCR using optimal conditions for permeabilization and PCR**

Cells	E1A positivity	
	+ Taq	- Taq
<b>Graham 293 cells</b>		
-cytospin preparations	60% positive	negative
-paraffin embedded section	40% positive	negative
<b>Infected A549 cell*</b>		
-cytospin preparations	n.d.	50% positive
-paraffin embedded sections	n.d.	70% positive
<b>Uninfected A549 cells</b>		
-cytospin preparations	0% positive	0% positive
-paraffin embedded section	0% positive	0% positive
<b>Tissue</b>		
<b>Guinea pig lung†</b>	1 or 2 positive cells/ section	negative
-Animals	2/2	0/2
-Sections	4/10	0/10
<b>COPD lungs</b>	1 or 2 positive cells/ sections	negative
-Cases	2/3	0/3
-Sections	2/6	0/6
<b>Lymph nodes††</b>	negative	negative
-Cases	0/3	0/3
-Sections	0/6	0/6
<b>Non-COPD lymph nodes</b>	††9 to 40 positive cells/ section	3 to 21 positive cells/section
-Cases	2/3	2/3
-Sections	4/6	4/6
<b>Uninfected guinea pig lung</b>	negative	negative
-Animals	0/2	0/2
-Sections	0/4	0/4

(\*) A549 cells infected with adenovirus 5 for 24 hours

(n.d.) not done

(†) Guinea pig latently infected with adenovirus type 5

(††) from COPD patients

(†††) see Table 5 for details

**Table 5.** Comparison of detection of E1A DNA in lymph nodes from non-COPD patients by indirect *in situ* PCR and standard *in situ* hybridization (ISH)

	# of cells with nuclear staining		
	indirect <i>in situ</i> PCR		standard ISH
	+ Taq	- Taq	
<b>E1A positive (block A)</b>			
slide 1	12	3	n.d.
slide 2	22	5	n.d.
slide 3	n.d	n.d.	2
<b>E1A positive (block B)</b>			
slide 1	9	6	n.d
slide 2	40.	21	n.d
slide 3	n.d	n.d.	4
<b>E1A negative (block C)</b>			
slide 1	0	0	n.d.
slide 2	0	0	n.d.
slide 3	n.d.	n.d.	0

(n.d.) not done

Amounts of carbon and other environmental contaminants (block C > block A > block B)

**Table 6. Summary of the detection of amplified E1A DNA by direct *in situ* PCR**

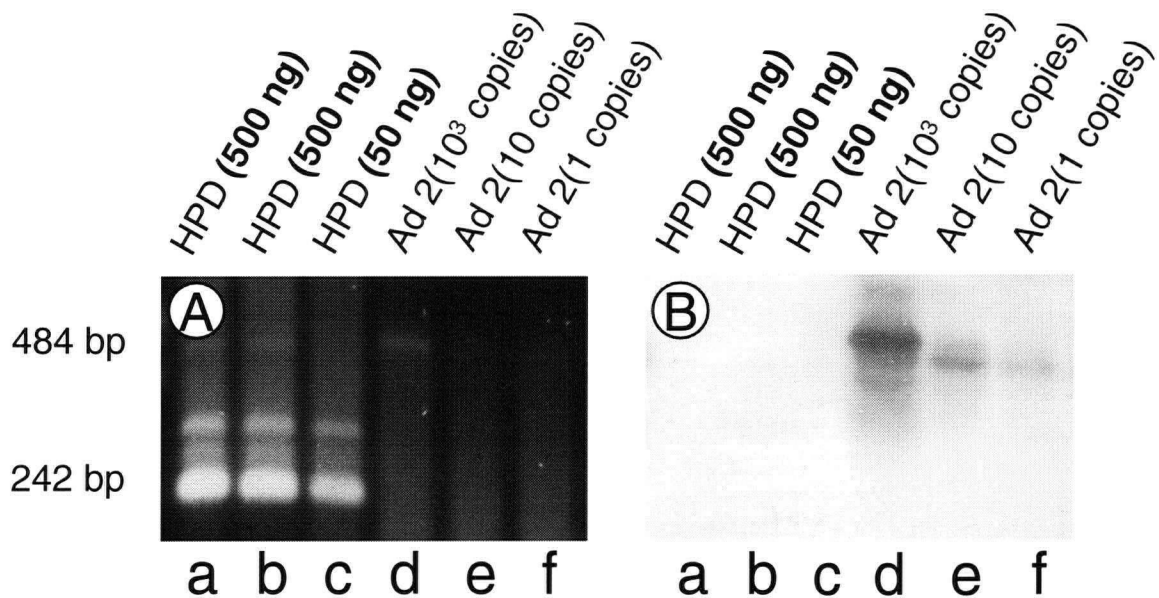
Cells	E1A positive cells	
	+ primers	- primers
<b>Graham 293 cells</b>		
-cytospin preparations (n=2)	70% positive	20% positive
-paraffin embedded section (n=2)	50% positive	1% positive
<b>Infected A549 cells*</b>		
-cytospin preparations (n=1)	100% positive	50% positive
-paraffin embedded sections (n=1)	100% positive	40% positive
<b>Uninfected A549 cells</b>		
-cytospin preparations (n=1)	20% positive	5% positive
-paraffin embedded sections (n=1)	negative	negative
<b>Tissue</b>		
Guinea pig lungs† (n=1)	4 positive cells/ section	negative
COPD lung (n=1)	1 positive cell/ section	negative
COPD lymph node (n=1)	n.d	n.d.
Uninfected guinea pig lung (n=1)	negative	negative

(n) is the numbers of slides used

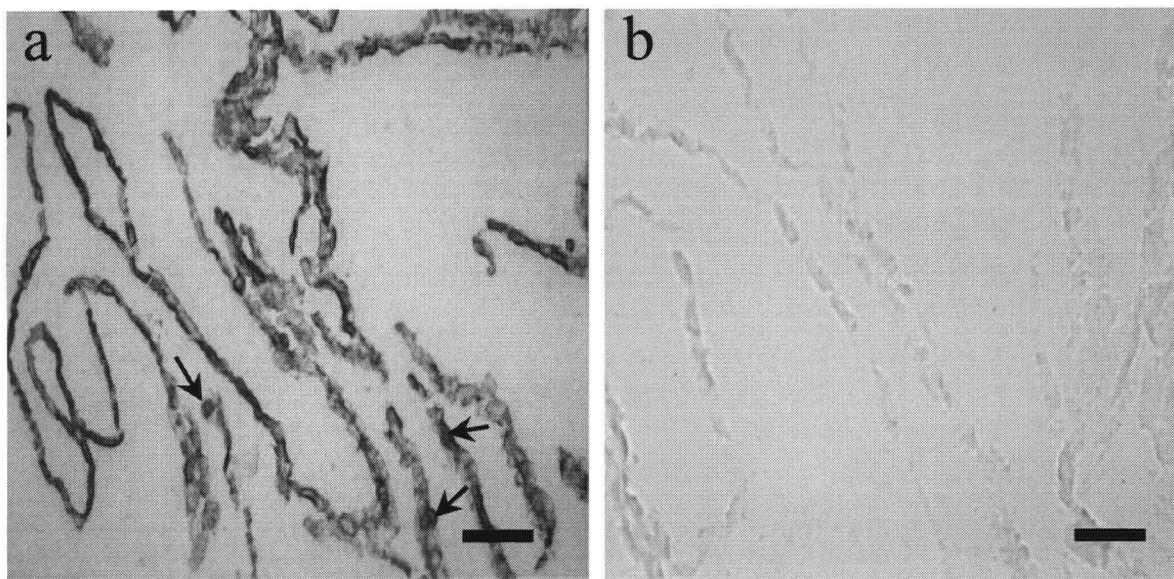
(\*) A549 cells infected with adenovirus type 5 for 24 hours

(†) Guinea pig latently infected with adenovirus type 5

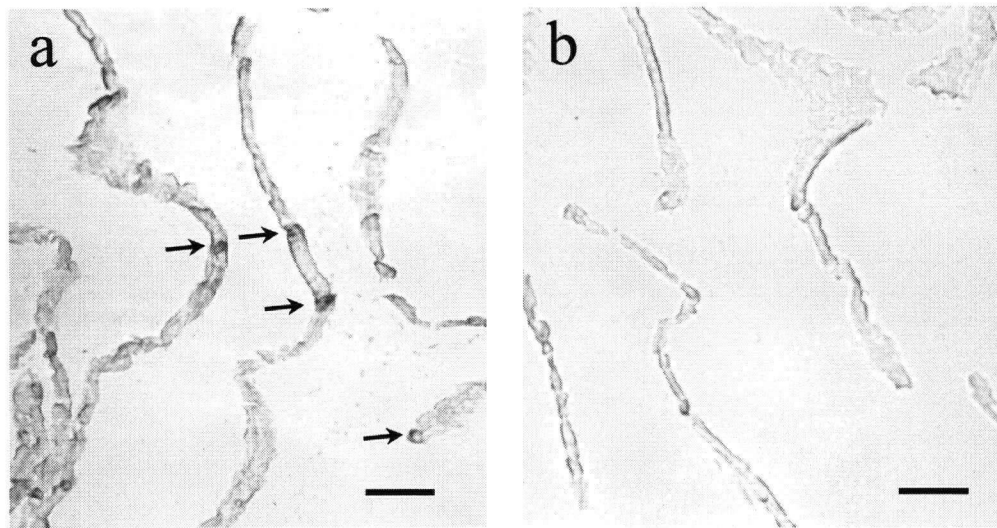
(n.d.) not done



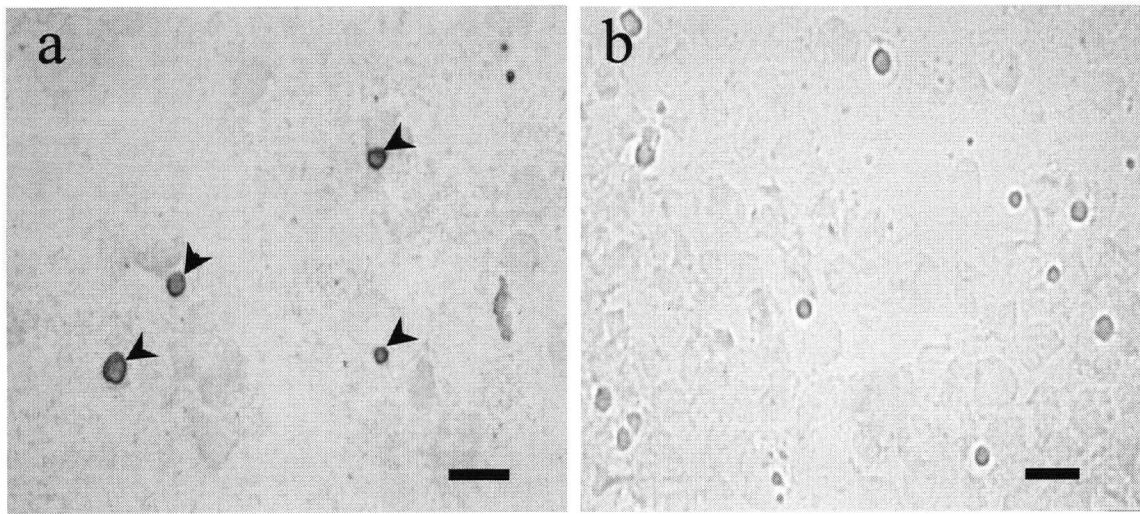
**Fig. 1.** Specificity of biotinylated E1A probe. Adenovirus 2 DNA (Ad 2) and human placental DNA (HPD) were used as templates to amplify E1A and HLA-DQ $\alpha$  DNA by solution phase PCR, respectively. **A)** Agarose gel of ethidium bromide stained E1A and HLA-DQ $\alpha$  PCR products. A 242 bp HLA-DQ $\alpha$  band is observed when 500 or 50 ng of human placental DNA were used as template for amplification (lanes **a**, **b**, and **c**). A 484 bp E1A band is seen when 10<sup>3</sup> copies of adenovirus 2 DNA were used as a template for amplification (lane **d**). **B)** Southern blot of E1A and HLA-DQ $\alpha$  PCR products hybridized with biotinylated E1A probe which was detected by streptavidin-alkaline phosphatase staining. Biotinylated E1A probe hybridized exclusively with 484 bp amplification product of adenovirus 2 E1A DNA. The intensity of the E1A band was greater when 10<sup>3</sup> copies of adenovirus 2 DNA (lane **d**) were used as template compared to 10 (lane **e**) or 1 (lane **f**) copies of templates. E1A probe did not hybridize with 242 bp PCR product of HLA-DQ $\alpha$  gene (lanes, **a**, **b**, and **c**).



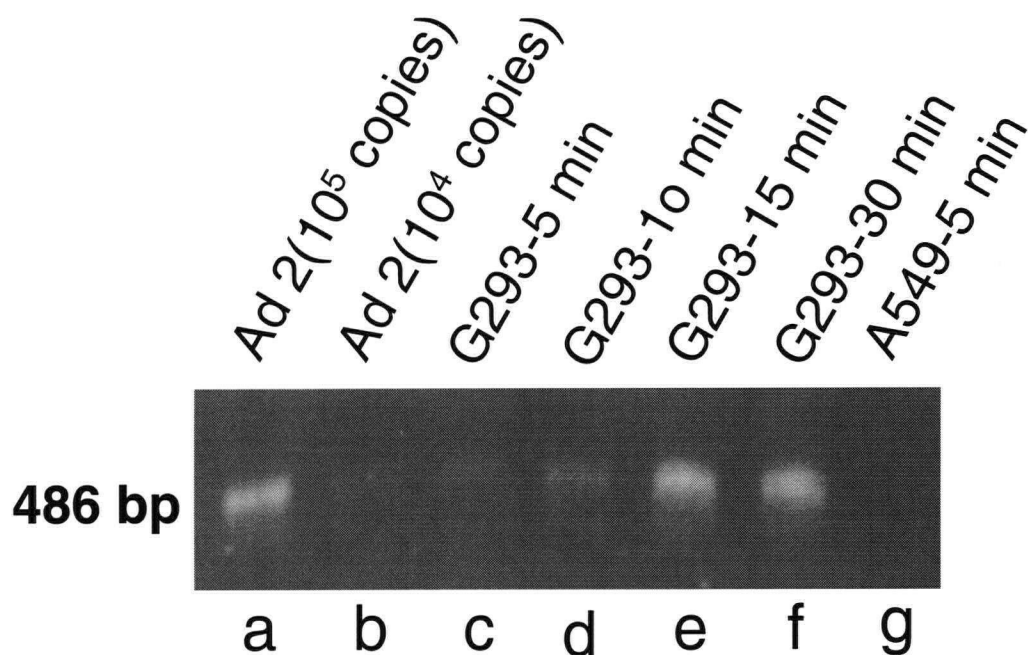
**Fig. 2.** Indirect *in situ* PCR on paraffin embedded sections of Graham 293 cells using 2 mM  $\text{MgCl}_2$  and 40 cycles of *in situ* E1A amplification showing diffuse background staining. Prior to indirect *in situ* PCR, paraffin embedded sections were digested with 1 mg/ml pepsin in 0.2 N HCl at room temperature for 15 minutes. *In situ* amplification was followed by *in situ* hybridization with biotinylated E1A probe. **a)** A diffuse signal is in the nuclei (arrows) and cytoplasm of the cells. Note that cytoplasmic signal is evident in all cells. **b)** No hybridization signal is evident on the adjacent section where Taq polymerase was omitted from the PCR mixture. Scale equals 25  $\mu\text{m}$ .



**Fig. 3.** Indirect *in situ* PCR detection of E1A DNA in paraffin embedded sections of Graham 293 cells under optimal PCR and permeabilization conditions. Prior to E1A *in situ* amplification (30 cycles) using 2 mM MgCl<sub>2</sub>, paraffin embedded sections had been digested with 1 mg/ml pepsin in 0.2 N HCl at room temperature for 10 min. **a)** E1A products in the nuclei (**arrows**) are detected after *in situ* hybridization with biotin-labeled E1A probe. **b)** No hybridization signal is evident on the negative control which is the adjacent section with Taq polymerase omitted from the PCR mixture. Bar equals 10 μm.

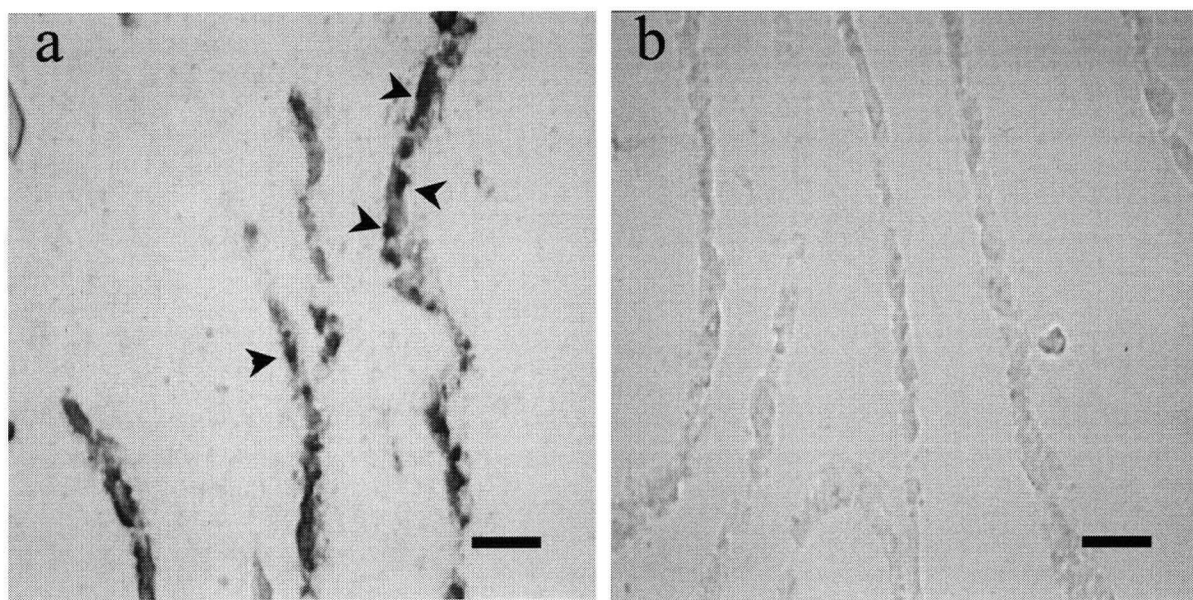


**Fig. 4.** Localization of E1A DNA in cytospin preparations of Graham 293 cells by indirect *in situ* PCR under optimal PCR and permeabilization conditions. **a)** Strong nuclear staining (**arrowheads**) is evident by *in situ* hybridization with biotinylated E1A probe after digestion with 50  $\mu\text{g/ml}$  proteinase K for 5 min at 37°C followed by 30 cycles of *in situ* E1A DNA amplification using 2 mM  $\text{MgCl}_2$  in the presence of Taq polymerase. **b)** Negative control. *In situ* hybridization with biotinylated E1A probe after *in situ* amplification in the absence of Taq polymerase shows no staining. Bar equals 15  $\mu\text{m}$ .

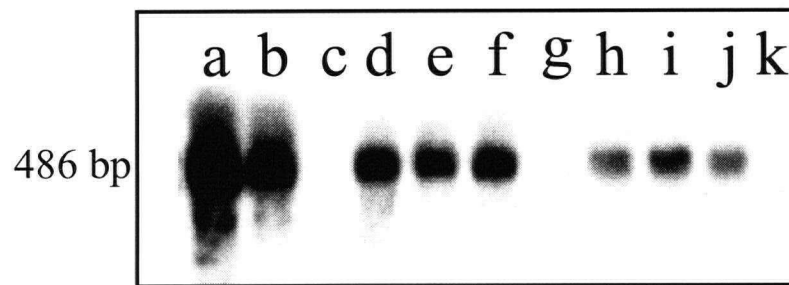


**Fig. 5.** Effect of proteinase K permeabilization time on the diffusion of amplified E1A product from cytospin preparations of Graham 293 cells. To demonstrate diffusion of amplified E1A product from cytospin preparations of Graham 293 cells (G 293) into the PCR mixture, the PCR solution overlying the section was recovered and subjected to 1% agarose gel electrophoresis. No E1A band is observed when Graham 293 cells was permeabilized with 50  $\mu$ g/ml proteinase K at 37° C for 5 min (lane **c**). A weak E1A band (lane **d**) is evident when permeabilization was extended to 10 minutes, suggesting minor diffusion of amplified E1A product. A strong E1A band is evident when permeabilization of Graham 293 cells was extended to 15 or 30 minutes (lane **e** and **f**, respectively) indicating greater diffusion of amplified E1A product compared to 10 minutes protease treatment (lane **d**). Amplifying solution recovered from a cytospin preparations of uninfected A549 cells that were permeabilized with 50  $\mu$ g/ml proteinase K at 37° C for 5 minutes was used as a negative control for E1A DNA contamination of the PCR mixture. No E1A band is evident when amplifying solution from uninfected A549 cells was subjected to gel electrophoresis (lane **g**). As a positive control, adenovirus 2 DNA was used as a template to amplify E1A DNA by solution phase PCR. Strong E1A band (lane **a**) is evident when  $10^5$  copies of Ad2 DNA was used as a template for amplification.

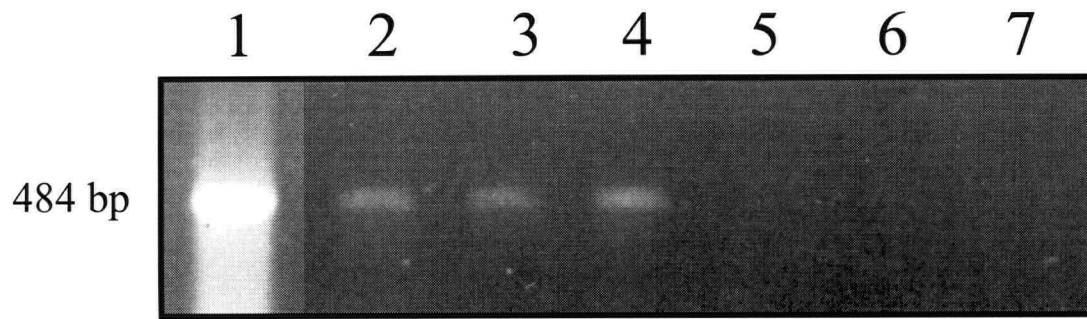




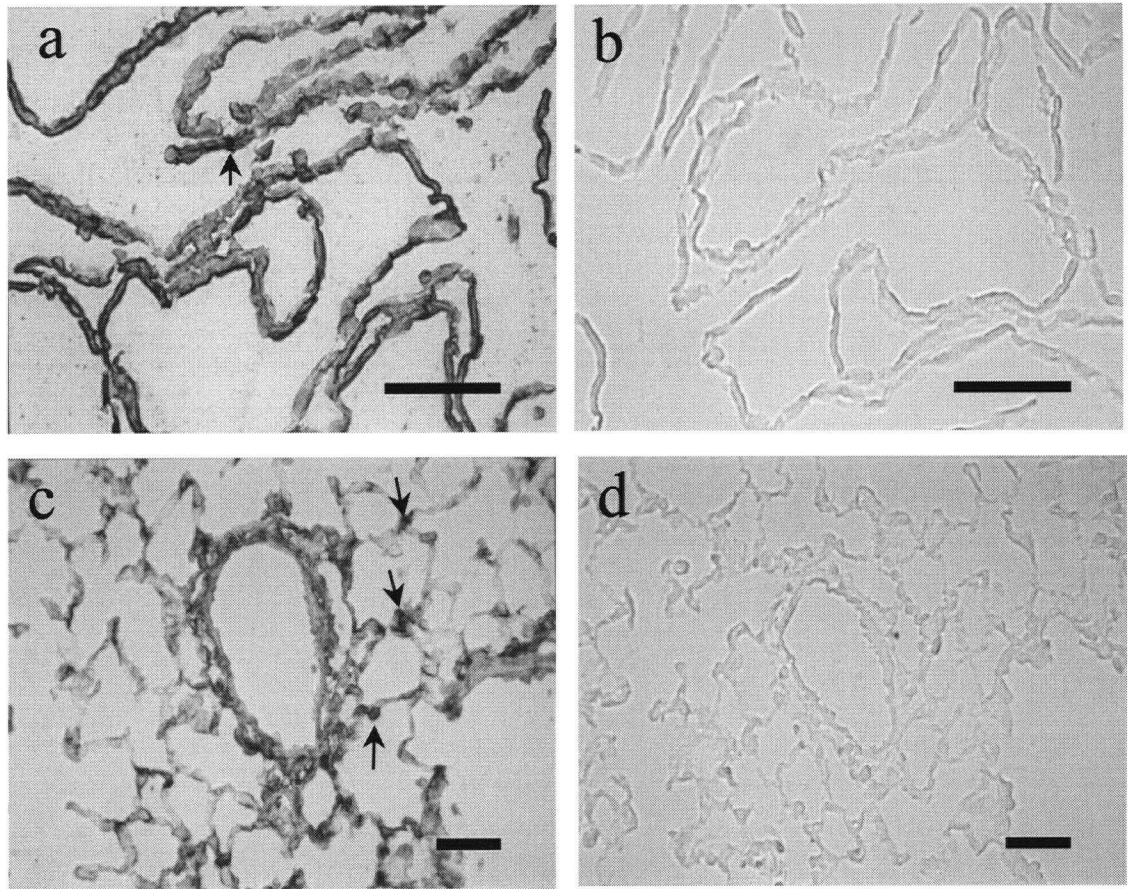
**Fig. 6.** Effects of pepsin digestion (1 mg/ml in 0.2 N HCl) at 37°C for 10 minutes prior to indirect *in situ* PCR on paraffin embedded sections of Graham 293 cells. **a)** Strong nuclear and cytoplasmic (**arrowheads**) staining are evident in all cells after 30 cycles of *in situ* E1A amplification followed by *in situ* hybridization with biotinylated E1A probe. Note that strong digestion damaged the cell architecture. **b)** No staining was observed on adjacent section where Taq polymerase was omitted from the PCR mixture. Bar equals 25  $\mu$ m.



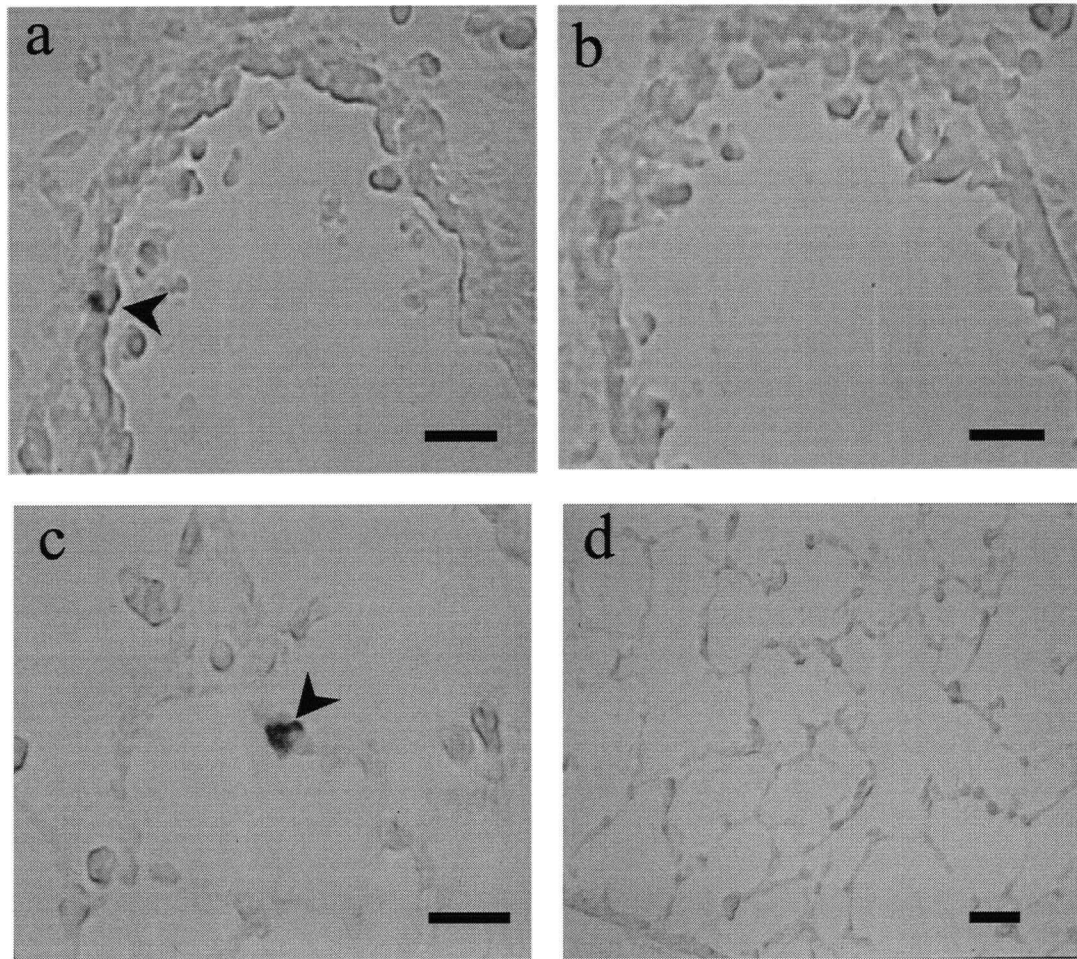
**Fig. 7.** Effect of pepsin digestion on the diffusion of amplified E1A product from paraffin embedded sections of Graham 293 cells. Southern hybridization analysis was used to demonstrate limited diffusion of amplified E1A product from Graham 293 cells into the PCR solution. E1A band of expected size (486 bp) was visualized after hybridization of membrane with  $^{32}\text{P}$ -labeled E1A probe. Pretreatment of paraffin embedded sections of Graham 293 cells with 1 mg/ml pepsin, at room temperature for 15 minutes prior to indirect *in situ* PCR gave a specific E1A band on the membrane (lane **d**, **e**, and **f**). Note that the E1A band intensity was decreased when pepsin digestion was reduced to 10 minutes (lanes, **h**, **i**, and **j**). In negative controls, paraffin embedded sections of uninfected A549 cells that had been digested with 1 mg/ml pepsin in 0.2 N HCl at room temperature for 10 or 15 minutes prior to indirect *in situ* PCR amplifying solutions do not show any band (lane **g** and **k**, respectively). Strong E1A band is evident when 10 or 1 copies of adenovirus 2 DNA were used as template to amplify E1A DNA by solution phase PCR (lane **a** and **b**, respectively). No E1A band is evident when adenovirus 2 DNA was omitted from the amplifying solution of solution phase PCR (lane **c**).



**Fig. 8.** Ethidium bromide stained 1% agarose gel electrophoresis of PCR products recovered in the PCR solution after *in situ* E1A PCR on paraffin embedded sections. To test the efficiency of solution phase PCR to amplify E1A DNA on paraffin embedded sections of cell cultures, the PCR solution was spiked with  $10^5$  copies of adenovirus 2 DNA. A 484 bp E1A band was evident when amplifying solution from Graham 293 cells (lane **2**) was subjected to gel electrophoresis. E1A band was also evident when amplifying solutions from uninfected A549 cells (lane **3**) and uninfected guinea pig lungs (lane **4**) were subjected to gel electrophoresis. The intensity of E1A band was the same in all three cases (lanes **2**, **3**, and **4**). Amplifying solution on a slide preparation with no section that had been spiked with  $10^5$  copies of adenovirus 2 DNA showed a corresponding band (lane **1**) of greater intensity than that from paraffin embedded sections (lanes, **2**, **3**, and **4**). No band was observed when Taq polymerase was omitted from the adenovirus 2 spiked-PCR solution on paraffin embedded sections of Graham 293 cells (lane **5**), uninfected A549 cells (lane **6**), or uninfected guinea pig lungs (lane **7**).

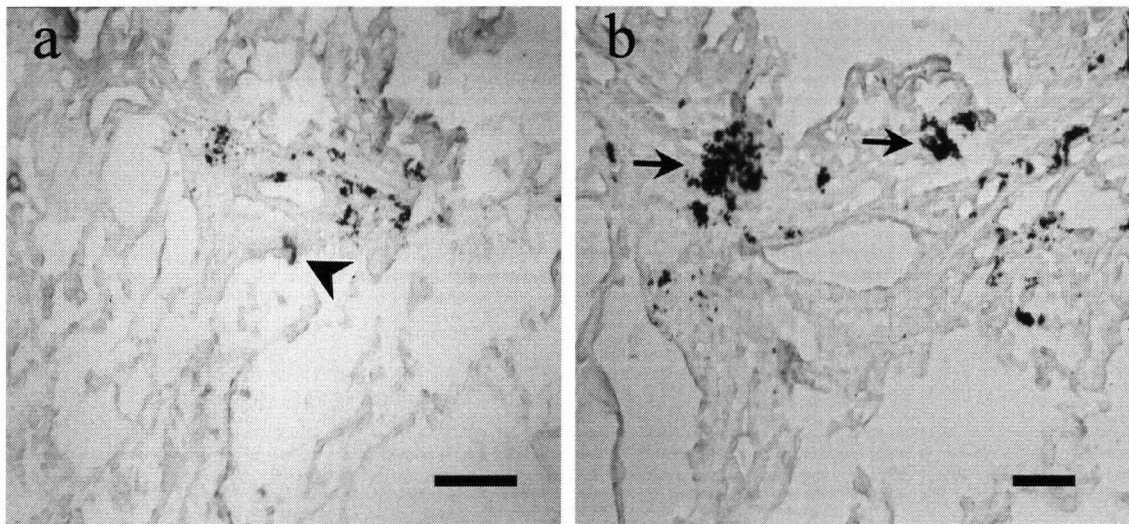


**Fig. 9.** Detection of nonspecific binding of the amplified E1A product generated in the solution phase to the paraffin embedded sections. *In situ* hybridization with biotinylated E1A probe after E1A DNA amplification in the presence of  $10^5$  copies of adenovirus 2 DNA yielded nuclear (**arrows**) and nonspecific cytoplasmic staining in paraffin embedded sections of Graham 293 cells (panel **a**) and uninfected guinea pig lungs (panel **c**). As control, the adjacent sections were also subjected to the same adenovirus spiked PCR. No staining was observed on adjacent control sections (panels, **b** and **d**) when Taq polymerase was omitted from the PCR mixture. Bar represents 50  $\mu$ m.

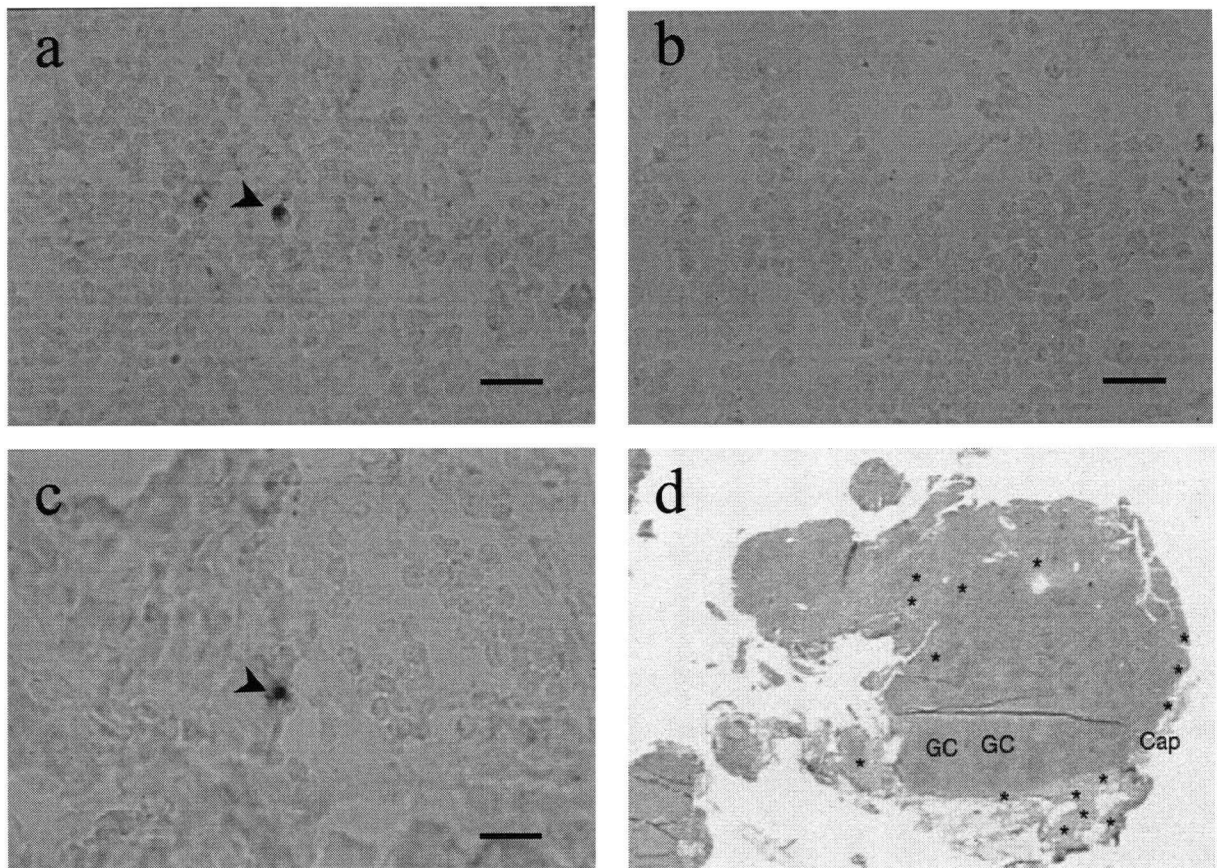


**Fig. 10.** Localization of E1A DNA in paraffin embedded serial sections of latently infected guinea pig lungs by indirect *in situ* PCR. *In situ* hybridization with biotinylated E1A probe after *in situ* E1A DNA amplification reveals nuclear staining (**a**) in an epithelial cell lining bronchiole (**arrowhead**) and in (**c**) a type II alveolar epithelial cell (**arrowhead**). Adjacent sections (**b** and **d**) to those shown in **a** and **c**, respectively, were treated with the same PCR mixture in the absence of Taq polymerase. No signal is evident after hybridization with biotinylated E1A probe. Bar represents 15  $\mu$ m.

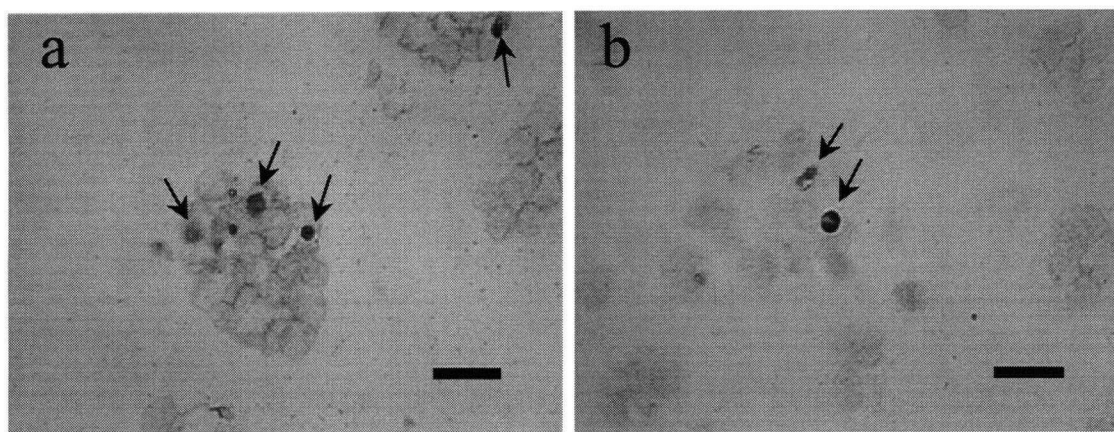




**Fig. 11.** Indirect *in situ* PCR localization of E1A DNA in paraffin embedded serial sections of lung from COPD patients. **a)** Nuclear staining is evident in alveolar epithelial cell (**arrowhead**) after *in situ* E1A DNA amplification followed by *in situ* hybridization with biotinylated E1A probe. **b)** When Taq polymerase was omitted from the PCR mixture, no hybridization signal was evident on the adjacent section. Note that carbon and other unidentified contaminants (**arrows**) are present on this section. Bar represents 30  $\mu\text{m}$ .

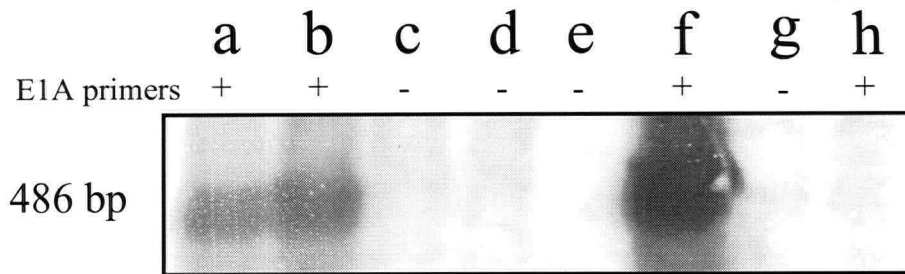


**Fig. 12.** Localization of E1A DNA in paraffin embedded sections of lymph nodes from non-COPD patients by indirect *in situ* PCR. **a)** *In situ* hybridization with a biotinylated E1A probe after *in situ* E1A DNA amplification reveals nuclear staining in an isolated cell around the germinal centre (arrowhead). **b)** When Taq polymerase was omitted from the PCR mixture on the adjacent section, this positive cell was not stained. **c)** Nuclear staining is evident in a cell near the capsule of lymph node (arrowhead) after *in situ* E1A DNA amplification followed by *in situ* hybridization with biotinylated E1A probe. **d)** H & E staining of the adjacent section to **a**, **b** and **c** showing location of capsule (Cap), germinal centers (GC) and E1A DNA nuclear staining (\*) after indirect *in situ* PCR. Bar equals 15  $\mu$ m.

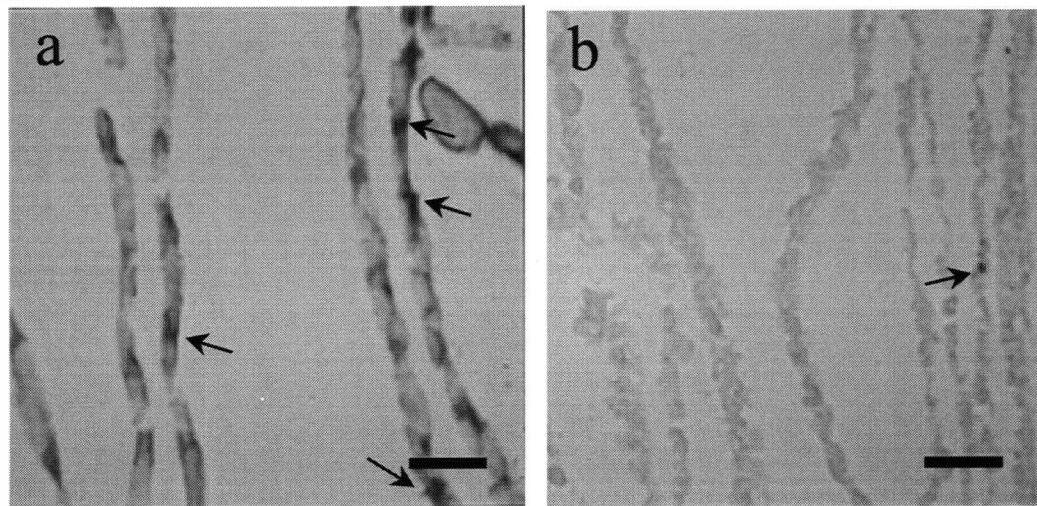


**Fig. 13.** Direct *in situ* PCR localization of E1A DNA in cytospin preparations of Graham 293 cells. Biotin-labeled products were visualized by streptavidin-alkaline phosphatase reaction. **a)** Strong nuclear staining (**arrows**) is evident when E1A primers were included in the PCR mixture. Approximately 70% of the cells showed nuclear staining. **b)** False positive nuclear signals (**arrows**) are evident when E1A primers were omitted from the PCR mixture. Approximately 20% of the cells show false positive staining. Bar equals 15  $\mu\text{m}$ .

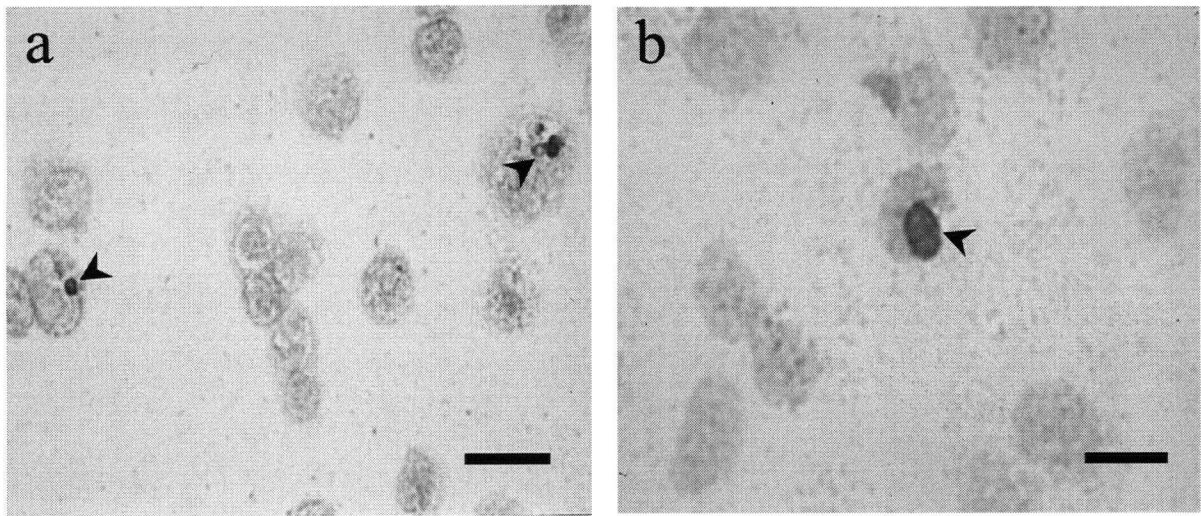




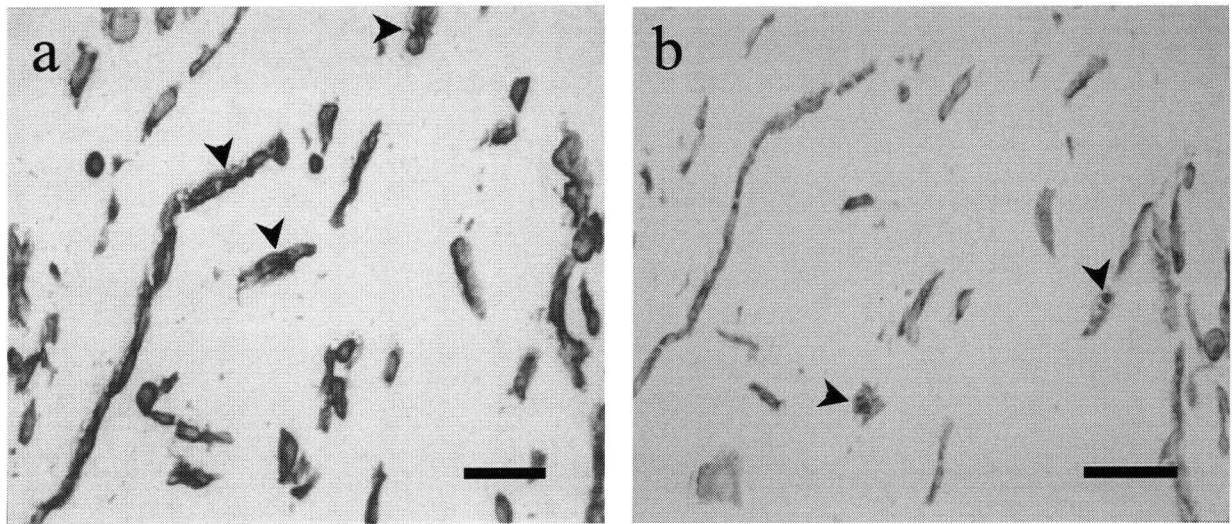
**Fig. 14.** Non-isotopic detection of amplified E1A products recovered after direct *in situ* PCR on cytospin preparations. Non-isotopic detection of biotinylated E1A DNA on Southern blot was used to demonstrate diffusion of amplified E1A products from Graham 293 and adenovirus infected A549 cells. The PCR products obtained with (+) and without (-) inclusion of E1A primers are shown. A 486 bp E1A band is evident when the recovered amplifying solutions from Graham 293 cells were subjected to streptavidin-alkaline phosphatase (SAAP) staining (lanes **a** and **b**). No band is evident when E1A primers were omitted from the PCR mixture (lanes **c** and **d**). Strong E1A band is visible when amplifying solution from adenovirus 5 infected A549 cells were subjected to SAAP staining (lane **f**). In the absence of E1A primers from the PCR mixture on adenovirus infected A549 cells, no E1A band is visible (lane **g**). As negative controls, amplifying solutions containing primers (lane **h**) and without E1A primers (lane **e**) from uninfected A549 cells do not show any band. Each lane represents PCR solution from a separate preparation.



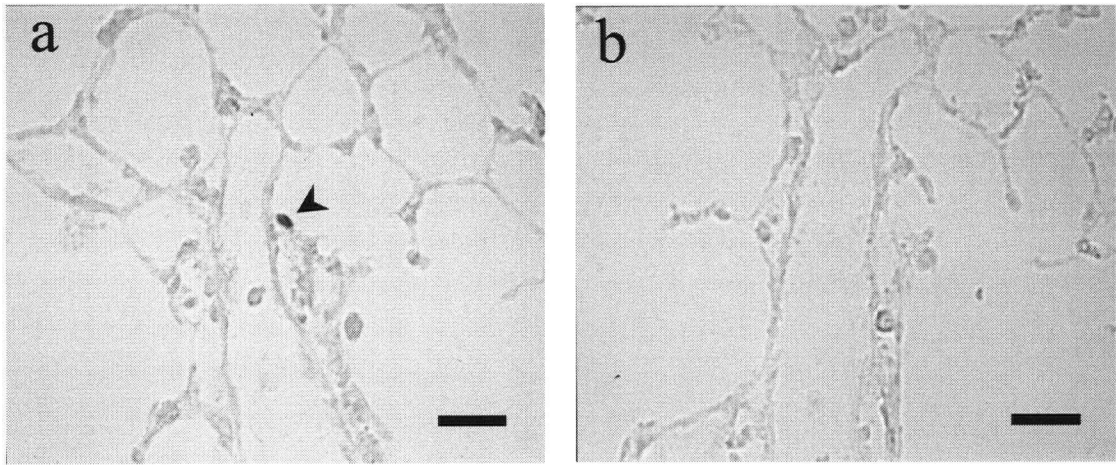
**Fig. 15.** Direct *in situ* PCR to detect E1A DNA in paraffin embedded sections of Graham 293 cells. Biotin labeled products are visualized by streptavidin-alkaline phosphatase reaction. **a)** Nuclear signal is evident in 50% of the cells (**arrows**) when E1A primers were included in the PCR mixture. **b)** False positive signal (**arrow**) is evident when E1A primers were omitted from the PCR mixture on the adjacent serial section. False positive signal is present in approximately 1% of the cells. Bar equals 25  $\mu\text{m}$ .



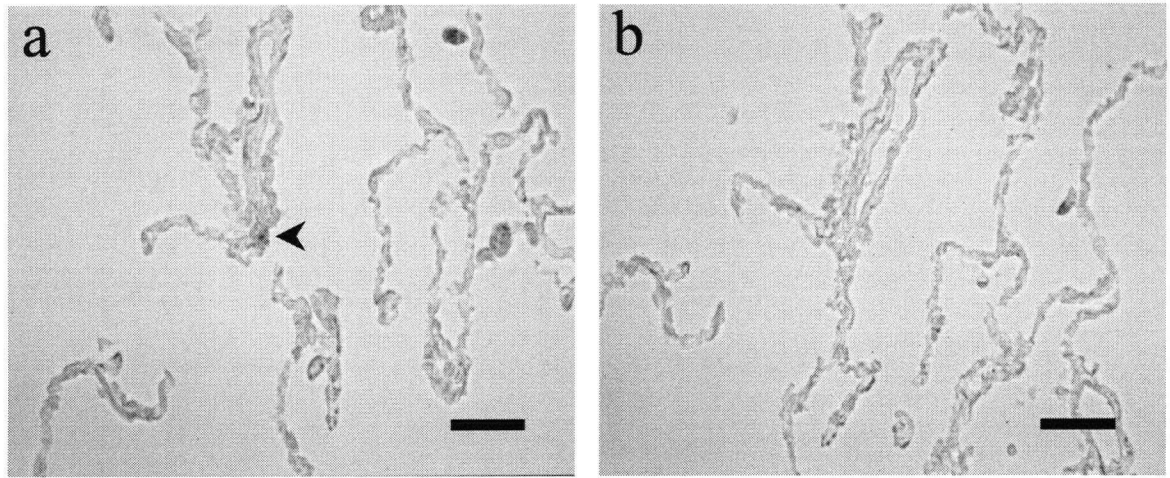
**Fig. 16.** Direct *in situ* PCR on cytospin preparations of uninfected A549 cells showing artifactual nuclear signals. Biotin labeled products are visualized by streptavidin-alkaline phosphatase staining. **a)** Note artifactual nuclear signal in nuclei of uninfected cells (**arrowheads**). False positive signal is present in approximately 20% of the cells. **b)** In the absence of the E1A primers from the PCR mixture, false positive signals are less frequent (**arrowhead**). Approximately 5% of the cells show evidence of nonspecific nuclear staining. Bar equals 15  $\mu$ m.



**Fig. 17.** Direct *in situ* PCR on paraffin embedded sections of adenovirus infected A549 cells showing nonspecific staining in the absence of E1A primers. **a)** Strong nuclear (**arrowheads**) and cytoplasmic staining are evident in all cells when E1A primers were included in the PCR mixture. **b)** Artifactual nuclear (**arrowheads**) and cytoplasmic signals are evident when E1A primers were omitted from the PCR mixture. Approximately, 50% of the cells show false positive staining. Bar equals 50  $\mu$ m.



**Fig. 18.** Direct *in situ* PCR localization of E1A DNA in paraffin embedded sections of latently infected guinea pig lung. Following *in situ* E1A amplification, biotin-labeled products were visualized by streptavidin-alkaline phosphatase reaction. **a)** A nuclear signal is evident in one type II alveolar epithelial cell (**arrowhead**) when E1A primers were included in the PCR mixture. **b)** In the absence of E1A primers from the PCR mixture on the adjacent section, no signal is evident. Bar equals 25  $\mu\text{m}$ .



**Fig. 19.** Direct *in situ* PCR to detect E1A DNA in paraffin embedded sections of lung from a COPD patient. After *in situ* amplification, biotin-labeled products were visualized by streptavidin-alkaline phosphatase staining. **a)** Nuclear staining is evident in an alveolar epithelial cell (**arrowhead**) when E1A primers were included in the PCR mixture. **b)** No signal is evident on an adjacent section when *in situ* amplification was performed without E1A primers. Bar equals 50  $\mu$ m.