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Genome Res. 1994 4: 89-96

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In this study we examine the factors that lead to nonspecific DNA synthesis during in situ PCR and solution-phase PCR. It was shown that primer-independent DNA synthesis can produce an intense signal during in situ PCR. This primer-independent pathway was apparently the result of the repair of DNA gaps induced by the heat treatment of the paraffin embedded tissue sections. This nonspecific signal could be eliminated by blocking gap repair with dideoxy-TTP, avoiding heat treatment, or DNase pretreatment. The primer-independent signal was also influenced by the length and mode of fixation and the sample tissue itself. Elimination of the primer-independent signal and the use of viral primers in tissues that did not contain the virus showed that nonspecific DNA synthesis could be eliminated by the hot start modification. Primer oligomerization did not produce a signal during in situ PCR, even when it occurred robustly in the amplifying solution. Generation of the primer-independent signal in solution-phase PCR with purified DNA required a cross-linking fixative, heating, the addition of bovine serum albumin, and intact protein–DNA cross-links.

The PCR methodology has had an enormous impact on molecular biology. However, the prerequisite DNA extraction precludes direct cellular localization of the amplified product. Although DNA can be localized by in situ hybridization, the relatively high detection threshold of ~10 copies per cell limits its utility.[1,2] PCR in situ hybridization, a process by which DNA is amplified in intact cells and then detected by in situ hybridization with an internal, labeled probe, permits the cellular localization of one target copy per cell.[1–4] Although it is an effective tool, the need for a hybridization step makes PCR in situ hybridization a relatively time-consuming and technically demanding procedure. Although direct incorporation of a reporter molecule has been reported with in situ PCR,[2,5] to assure target-specific DNA amplification it is essential to understand the potential pathways for nonspecific DNA synthesis that may occur in the cell.

Prior work has demonstrated several important differences in the optimal conditions and dynamics of in situ PCR versus solution-phase PCR. Ethanol and acetone fixation, which are successfully used for solution-phase PCR, do not allow for optimal in situ PCR because of migration of the amplified product out of the cell.[6] Prolonged formalin fixation appears to create a migration barrier that prevents this problem. Target-specific amplification with in situ PCR consistently required higher concentrations of $MgCl_2$ than for solution-phase PCR. Bovine serum albumin (BSA) enhances DNA synthesis during in situ PCR because it blocks adsorption of the enzyme *Taq* polymerase to the microscopic slide.[6] However, it is apparent that as in solution-phase PCR, in situ PCR is subject to side reactions that compete with target-specific amplification. Nonspecific incorporation of digoxigenin dUTP has been reported with in situ PCR using unheated cytospin preparations.[3,6] This nonspecific incorporation was blocked by the hot start modification to PCR and presumably reflects mispriming.[2,3,6]

Previous work has documented nonspecific DNA synthesis during in situ PCR in tissue sections. It was demonstrated that unlike cytospin preparations, the nonspecific DNA synthesis during in situ PCR in tissue sections was not eradicated by the hot start maneuver.[2,5] The basis for this difference is unclear. Nonspecific DNA synthesis in tissue sections can be blocked by overnight digestion in RNase-free DNase, which permits target-specific incorporation of the reporter molecule into amplified cDNA with reverse transcriptase (RT) in situ PCR.[2,5] A better understanding of the nonspecific pathways for DNA synthesis during in situ PCR is needed to assure target-specific DNA and cDNA amplification in tissue sections. The purpose of this study was to determine the causes of nonspecific DNA synthesis during in situ PCR and solution-phase PCR.

MATERIALS AND METHODS

Tissue Preparation

Four-micron sections of paraffin-embedded human liver, vulvar, tonsilar, and lymph node tissues was placed on silane-coated glass slides (ONCOR, Gaithersburg, MD). Tissue samples were fixed in 10% neutral buffered formalin for 4 hr, 6 hr, 8 hr, 15 hr, 48 hr, or 1 week. Protease digestion was done using pepsin at 2 mg/ml (Life Technologies, Gaithersburg, MD) in 0.01 N HCl at room temperature. Three serial sections were placed on each

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slide so that adjustments to a given variable could be compared under identical reaction conditions. Sections were deparaffinized in xylene for 5 min, washed in 100% ethanol for 5 min, and air-dried.

In Situ PCR

In situ PCR refers to the technique whereby a (digoxigenin) labeled nucleotide is directly incorporated into the amplified product in intact cells. After deparaffinization and protease digestion, the following reaction mixture (25 μl) was added to each slide: 2.5 μl of PCR buffer II (GeneAmp Kit, Perkin-Elmer Corporation), 4.5 μl of 25 mM $MgCl_2$ (4.5 mM final concentration), 4.0 μl of dNTP solution (final concentration of 200 μM each of dATP, dCTP, dGTP, and dTTP), 1.0 μl of 2% BSA, 0.4 μl of 1 mM digoxigenin dUTP solution (10 μM final concentration; Boehringer Mannheim, Indianapolis, IN)±1 μl of primer 1 and primer 2 (a variety of primers were used as stated in other parts of the text; the stock solution of each primer was 20 μM), 11 μl (or 13 μl if the primers were omitted) of water, and 0.6 μl of *Taq* polymerase (Perkin-Elmer Corporation, 5 U/μl). The solution was added to the slide, covered with one large polypropylene coverslip (Oncor Corporation), anchored with 2 small drops of nail polish, and placed in an aluminum foil "boat." This was placed on the block of the thermal cycler (TC 1, Perkin-Elmer Corporation), which was ramped to 80°C. At this temperature, the coverslip was overlaid with 1 ml of heated mineral oil. The DNA was denatured at 94°C for 3 min, followed by 20 cycles at 55°C for 2 min and 94°C for 1 min. After removal of the coverslip, sequential 5-min washes in xylene and 100% ethanol were done, and the slides air-dried. Detection of digoxigenin incorporated into the PCR product was done with the alkaline phosphatase-conjugated antidigoxigenin-labeled antibody (Boehringer Mannheim) at a 1:200 dilution as described previously.[2,3,5,6] The alkaline phosphatase-based colorimetric detection method used the chromogen nitroblue tetrazolium (NBT) which, in the presence of 5-bromo-4-chloro-3-indolylphosphate (BCIP), yields a dark blue precipitate as the marker for positive cells. The counterstain, nuclear fast red (ONCOR, Gaithersburg, MD), stains nuclei and cytoplasm pale pink. Tests were done in triplicate, and the scoring system for the signal was as follows: 0, 1+ (<25% of cells positive), 2+ (25–50% of cells positive), and 3+ (>50% of cells positive). Signal measurements were made without knowledge of the reaction conditions.

Dideoxy-TTP Blockage

To determine whether the nonspecific signal could be influenced by pretreatment with a solution that contained a dideoxynucleotide and *Taq* DNA polymerase, implying a DNA repair mechanism, tissues were incubated after protease digestion and prior to in situ PCR in a solution that contained 2.5 μl of PCR buffer, 4.5 μl of 25 mM $MgCl_2$ (4.5 mM final concentration), 4.0 μl of dNTP solution (final concentration of 50 μM each of dATP, dCTP, dGTP, and dTTP), 2.5 μl of a 10 mM dideoxy-TTP (final concentration of 1000 μM), 1.0 μl of 2% BSA, 9.7 μl of water, and 0.8 μl of *Taq* polymerase (Perkin-Elmer Corporation, 5 U/μl) for 30 min at 55°C. The dideoxy-TTP and other reagents were removed by sequential 5-min washes in xylene and ethanol and were then air-dried. In situ PCR was performed next using the conditions listed in the preceding paragraph.

DNase Digestion

To determine whether the nonspecific signal could be influenced by pretreatment with a solution that contained DNase, tissues were incubated after protease digestion and prior to in situ PCR in a solution that contained 1.0 μl of buffer (which contained 5 mM $MgSO_4$ and 100 mM sodium acetate), 8.0 μl of DEPC-treated water, and 1.0 μl of RNase-free DNase (10 U/μl, Boehringer Mannheim). To prevent drying, the solution was overlaid with a coverslip and the slides were placed in a humidity chamber at 37°C overnight. The DNase was removed by successive washes of 2 min in DEPC-treated water and 100% ethanol.

Solution-phase PCR

Nonspecific primer-independent DNA synthesis in solution-phase PCR was studied three ways. First, DNA was extracted and purified as described previously[2] from tonsilar tissues that were fixed for 15 hr in formalin either after freezing or embedding in paraffin. Second, purified salmon sperm DNA (Oncor Corporation) was fixed either in acetone, formalin, or 95% ethanol at times in the presence of 2.5 μg of BSA. After fixation for 4 hr, the DNA was purified using sodium acetate/ethanol precipitation.[2] Third, DNA was extracted from peripheral blood mononuclear cells isolated with a Ficoll gradient,[6] and treated with formalin, heat (68°C) and formalin, or heat, formalin, and BSA for 3 hr, and then precipitated. In each case, solution-phase PCR was done using 2.5 μg of DNA, 1.5 mM $MgCl_2$, 200 μM of each dATP, dCTP, dGTP, and dTTP, buffer (PCR buffer II, GeneAmp kit, Perkin-Elmer Corporation), and 1 μM [^{32}P]dCTP for 25 cycles. PCR-amplified DNA was separated by electrophoresis on a 1.5% agarose gel, transferred to a nitrocellulose filter, and analyzed by autoradiography as described previously.[2,6]

RESULTS

In Situ PCR: Primer-independent DNA Synthesis

To determine if primer-independent DNA synthesis was operative during in situ PCR, the procedure was done using paraffin-embedded liver, tonsilar, vulvar, and lymph node tissues with omission of the primers. Initial experiments were done using tissues fixed for 15 hr. For each tissue, an optimal (3+) signal was obtainable after 60–90 min of pepsin digestion.

Primer-independent Signal and Fixation Plus Protease Digestion Times

Protease digestion is needed after formalin fixation to create channels in the resultant protein–DNA cross-linked matrix to allow penetration of the PCR reagents. The effect of times of fixation and protease digestion on the primer-independent signal with in situ PCR was examined for the vulvar tissues. These data are compiled in Table 1. Note that no signal was evident after any fixation time if the protease digestion step was omitted. Furthermore, note the strong correlation between the times of formalin fixation and protease digestion needed to produce an optimal signal. Each of the triplicate results yielded the same data using

TABLE 1 Effect of Protease Digestion Time on the Primer-independent Signal during In Situ PCR as a Function of the Fixation Time in 10% Buffered Formalin

	Protease digestion time (min)[a]								
Fixation time	0	5	10	15	30	45	60	75	90
4 hr	0	1+	3+	2+	overdigested				
6 hr	0	0	1+	3+	2+	overdigested			
8 hr	0	0	0	0	1+	3+	–	–	–
15 hr	0	0	0	0	0	1+	1+	2+	3+
48 hr	0	0	0	0	0	0	1+	2+	3+
1 week	0	0	0	0	0	0	1+	1+	2–3+[b]

The signal was scored as follows: 0, 1+ (<25% of cells positive), 2+ (25–50% cells positive), and 3+ (>50% of cells positive). Signal measurements were made without knowledge of the reaction conditions.
[a](Protease) Pepsin (2 mg/ml) at room temperature. Overdigested refers to loss of tissue morphology with a concomitant loss of the in situ PCR signal.
[b]The 2+ signal was with pepsin, the 3+ signal with proteinase K digestion (1 mg/ml).

the broad 0–3+ categories (see Materials and Methods).

DNase Digestion and the Primer-independent Signal

To determine whether the primer-independent signal required an intact DNA template, the tissue sections were treated overnight in a solution that contained RNase-free DNase. The primer-independent signal could be eliminated under these conditions, but this loss of signal was also highly related to the times of fixation and protease digestion (Table 2). Note that for a given fixation time the protease time that resulted in an optimal signal yielded no signal after the DNase predigestion (data not shown). However, for less than optimal protease times, there was at times an enhanced signal for the DNase-digested section when compared with the other serial section present on the same glass slide that was not digested with DNase.

Dideoxy-TTP Pretreatment and the Pprimer-independent Signal

The primer-independent signal could represent repair of single-stranded gaps or nicks. Incorporation of a dideoxynucleotide in regions of DNA gaps could render such areas unavailable to digoxigenin incorporation during the PCR phase of the procedure. To test this hypothesis, we pretreated the sections after protease digestion in a solution that contained dideoxy-TTP (in a ratio of 20:1 with respect to dTTP as well as dATP, dCTP, and dGTP) and *Taq* polymerase (see Materials and Methods). After this pretreatment and removal of the dideoxy-TTP by washing, in situ PCR was performed in the absence of any primers. The results are compiled in Table 3. Two similarities with the data for the DNase pretreatment are evident: (1) At the protease time optimal for the primer-independent signal, the dideoxy pretreatment eliminated the nonspecific signal; (2) at suboptimal protease times (suboptimal defined by a signal <3+ with no pretreatment step), the dideoxy pretreatment at times enhanced the signal relative to the serial section on the same glass slide that was not pretreated with dideoxy-TTP (Fig. 1). Note that unlike the DNase experiments, a signal reemerges after dideoxy pretreatment with supraoptimal protease digestion times (supraoptimal defined by digestion times greater than that required to produce a 3+ signal with no pretreatment step). An alternative explanation for the elimination of the primer-independent signal with dideoxy pretreatment under the conditions defined above is that the polymerase activity of the *Taq* polymerase during the pretreatment step filled in the putative gaps and that this rather than dideoxy termination was responsible for the loss of the signal during the subsequent in situ PCR. To test this hypothesis, direct comparisons on the same glass slide were done of the pretreatment step with and without the 1000 μM of dideoxy-TTP under the optimal protease digestion conditions defined in Materials and Methods. The signal during in situ PCR that was completely blocked with dideoxy pretreatment was 3+ if the dideoxy-TTP was omitted from the pretreatment solution and replaced with 150 μM dTTP (data not shown), demonstrating that presence of the dideoxy nucleotide in the pretreatment step was crucial for the subsequent elimination of the primer-independent signal during in situ PCR.

TABLE 2 The Effect of Protease Digestion Time on the Primer-independent Signal during In Situ PCR with and without Overnight Digestion in RNase-free DNase

	Protease digestion time (min)				
Fixation time	15	30	45	60	90
8 hr					
no DNase digestion	0	1+	3+	2+	–
DNase digestion	2+	0	0	0	–
15 hr					
no DNase digestion	0	0	1+	1+	3+
DNase digestion	2+	2+	1+	0	0

Exonuclease Activity and Enhancement of Signal with Dideoxy Blockage

To address whether the signal evident with dideoxy pretreatment at sub- and supraoptimal protease digestion times was related to the 5′ and 3′ exonuclease activity of *Taq* polymerase during the pretreatment step, the experiments were repeated substituting the Stoffel frag-

TABLE 3 The Effect of Protease Digestion Time on the Primer-independent Signal during In Situ PCR with and without Pretreatment in Dideoxy-TTP

Fixation time	Protease digestion time (min) 5	10	15	20
6 hr				
no dideoxy treatment	0+	1+	3+	2+
dideoxy pretreatment	2+*	1+	0	3+
	Protease digestion time (min) 15	30	45	60
8 hr				
no dideoxy treatment	0	1+	3+	2+
dideoxy pretreatment	2+*	1+	0	2+

The range of the signal under these conditions varied from 1+ to 3+ in the triplicate experiments, whereas each result was 0 for the tissue section not pretreated with the dideoxy nucleotide.

ment, which lacks the exonuclease function, for the *Taq* enzyme. The pretreatment conditions with the Stoffel fragment were identical to the *Taq* polymerase dideoxy pretreatment, except the Stoffel buffer was substituted for PCR buffer II (each from Perkin-Elmer). No signal was evident during in situ PCR after the dideoxy pretreatment when the *Taq* polymerase was substituted by the Stoffel enzyme at optimal or suboptimal protease digestion times. However, a signal during in situ PCR equivalent with the Stoffel fragment to that noted with *Taq* polymerase-mediated dideoxy pretreatment was noted at supraoptimal protease digestion times (data not shown).

The Primer-independent Signal and Tissue Heating

The primer-independent pathway probably reflects repair of DNA gaps/nicks based, in part, on its elimination with dideoxy pretreatment. For embedding in paraffin, the tissue was treated in graded ethanol and xylene, and heated in paraffin wax at 65°C for 4 hr. We studied whether the heating and/or ethanol and xylene washes during paraffin embedding might induce formation of the putative gaps/nicks. Tissue sections from frozen vulvar and tonsilar biopsies were placed on silane slides and fixed for 4 or 15 hr in 10% buffered formalin. No primer-independent signal was evident in either tissue during in situ PCR (Fig. 2), even if the tissues were washed prior to PCR in ethanol and xylene (data not shown). However, all of the cells showed a signal when primers specific for β-globulin, present as two copies per cell, were used in the PCR step under optimal protease digestion conditions (data not shown). To determine whether the 4 hr heating at 65°C could induce the primer-independent signal in these frozen and then formalin-fixed tissues, the sections were heated in a dry oven at 65°C for 4 hr after fixation. A primer-independent signal was evident at optimal protease digestion time for each tissue after this heating step was performed (Fig. 2). This signal was evident even after 1 hr of heating (data not shown).

Primer-independent Signal in Solution-phase PCR

To determine whether the primer-independent pathway was operative during solution-phase PCR, total cellular DNA was extracted from fresh peripheral blood mononuclear cells isolated from a Ficoll gradient as identified previously,[6] and treated with 10% buffered formalin for 3 hr either at room temperature or at 68°C. The effect of BSA was also tested by adding 2 ng/μl of the protein to a sample of the DNA treated with formalin and heat. The DNA was then ethanol precipitated and subjected to PCR with direct incorporation of [^{32}P]dCTP (see Materials and Methods).

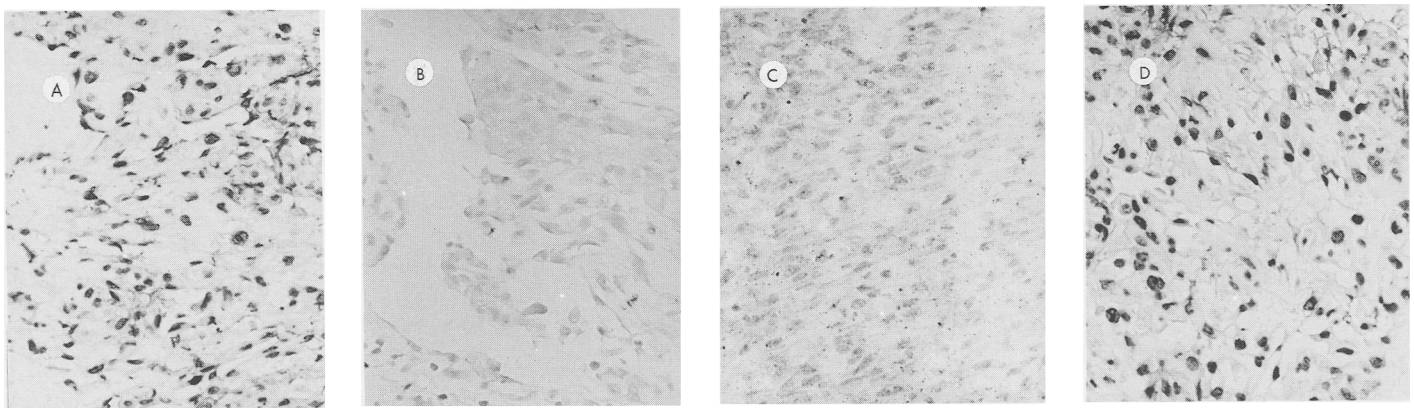

FIGURE 1 Effect of pretreatment with a dideoxy nucleotide on the primer-independent signal during in situ PCR. An intense signal was seen in most cells in this liver biopsy fixed in formalin for 8 hr with in situ PCR after 30 min of protease digestion (*A*). The signal was not evident if the protease digestion time was decreased to 10 min (*B*). The signal evident after 30 min of protease digestion was eliminated by pretreatment in a solution which contained dideoxy-TTP (*C*). However, a strong signal was seen after 10 min digestion if the in situ PCR was preceded by the dideoxy pretreatment (*D*).

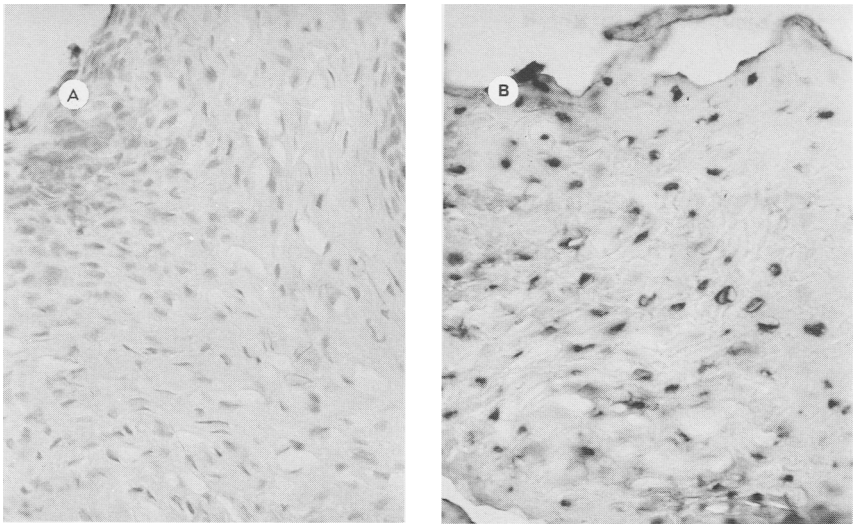

FIGURE 2 Temperature dependency of the primer-independent signal during in situ PCR. No primer-independent signal was noted after in situ PCR in this skin biopsy that was frozen, sectioned, and fixed in formalin (*A*). A signal was evident if the tissue section was heated at 65°C for 4 hr prior to in situ PCR (*B*).

As is evident from Fig. 3, a primer-independent signal required that the DNA be fixed in formalin at 68°C in the presence of BSA. Similarly, the DNA was extracted from the frozen, fixed, or paraffin-embedded formalin-fixed lymphoid tissues, ethanol precipitated, and also subjected to solution-phase PCR with [^{32}P]dCTP incorporation. A primer-independent signal was evident in the DNA extracted from the paraffin-embedded, fixed tissue but not the frozen, fixed tissue (data not shown).

To determine whether DNA–protein cross-linking by formalin was required for the primer-independent signal during solution-phase PCR, we used highly purified salmon-sperm DNA. The salmon sperm DNA (2.5 μg) was subjected to fixation for 4 hr in 95% ethanol, acetone (neither cross-link), or 10% buffered formalin; the addition of 2.5 μg of BSA with each fixative was also tested. The purified DNA, which according to the manufacturer is ~800 bp in size and was heated at 121°C during the processing, was subjected to solution-phase PCR in the absence of primers (Fig. 4). Note that a primer-independent signal at the expected size of 800 bp was only evident for the DNA fixed in formalin in the presence of BSA. Also note how the signal was lost from the DNA fixed in formalin with BSA after postdigestion in 1 mg/ml of proteinase K for 3 hr at 55°C; the protease was degraded prior to PCR by heating at 95°C for 10 min. Interestingly, formalin fixation prevented the nonspecific binding of the [^{32}P]dCTP to the BSA, presumably because of removal of the positive charged moieties on the protein.

Next, we attempted to determine the relative contribution of the nonspecific primer-dependent pathways (mispriming and primer oligomerization) to the signal evident with in situ PCR. The basis of these analyses included skin and tonsil tissues and primers that that amplify regions of the measles virus, cytomegalovirus, and Epstein-Barr virus. The tissues were not infected by these viruses based on the lack of the characteristic histologic changes and the negative results obtained with solution-phase PCR. Thus, any signal generated during in situ PCR with these primers would be nonspecific. Demonstration that nonspecific signal was eliminated completely with overnight DNase digestion, presupposing optimal protease pretreatment, allowed for the study of the contribution of primer oligomerization, as the DNase digestion should eliminate the possibility of mispriming. The use of any of the viral primer pairs listed above in tissues digested by DNase under optimal protease conditions did not allow for a signal in the cells. The experiments were done under cold start conditions to enhance the production of the primer dimers. To demonstrate that primer oligomerization-mediated DNA synthesis was occurring in the amplifying solution, it was retrieved and electrophoresed on a 1.5% agarose gel, and the DNA was transferred to a filter. DNA synthesis was demonstrated after detection of the incorporated digoxigenin, which revealed a smear from 50 to ~500 bp (data not shown). It was determined that a migration barrier may inhibit entry into the cell of the relatively small double-stranded primer–dimers but not of

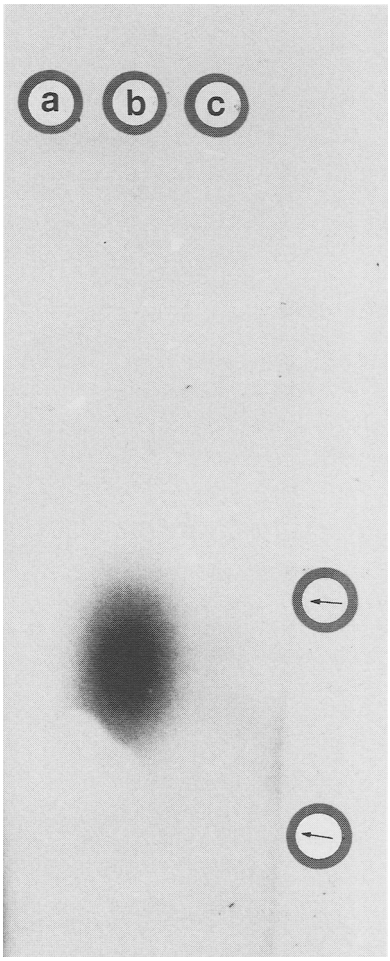

FIGURE 3 Primer-independent DNA synthesis during solution-phase PCR. The DNA was extracted from peripheral blood mononuclear cells and fixed for 3 hr in formalin at room temperature (lane *a*), in formalin and BSA at 68°C (lane *b*), or in formalin at 68°C for 3 hr (lane *c*), precipitated, and subjected to PCR with direct incorporation using [^{32}P]dCTP without primers. Note that primer-independent DNA synthesis is evident only after formalin fixation and heat in the presence of BSA. The arrows mark 700 (*top*) and 300 bp (*bottom*) as detected on the ethidium gel using a 100-bp ladder (Research Genetics).

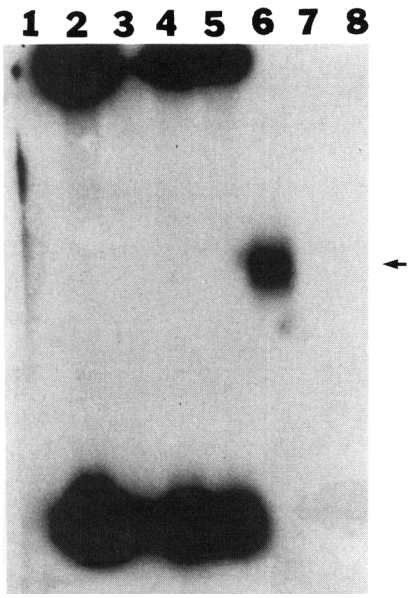

FIGURE 4 Variables necessary for primer-independent DNA synthesis during solution-phase PCR. Solution-phase PCR was done without primers using direct incorporation of [^{32}P]dCTP and salmon sperm DNA that was treated under the following conditions: no treatment (lane *1*), mixed with BSA (lane *2*), fixed in ethanol (lane *3*), fixed in acetone with BSA (lane *4*), fixed in ethanol with BSA (lane *5*), fixed in formalin with BSA (lane *6*), fixed in formalin with BSA but digested in proteinase K prior to PCR (lane *7*), and fixed in formalin (lane *8*). The arrow marks 800 bp as evident on the ethidium gel with the marker DNA. Note that a signal is evident at the expected size of the salmon sperm DNA (800 bp) if the salmon sperm DNA was fixed in formalin with BSA (lane *6*). This signal is lost with proteinase K treatment (lane *7*).

the individual single-stranded primers. Primer oligomerization also appears to be severely limited within the confines of the DNA–protein meshwork present in the formalin-fixed cell.

To test the role of mispriming during in situ PCR it was necessary to eliminate the nonspecific primer-independent signal without compromising the integrity of the DNA to permit annealing of the viral primers with the nontarget human DNA. This could be achieved either by using frozen, fixed tissue or paraffin-embedded tissue that was pretreated with optimal protease digestion time and dideoxy-TTP blockage. Both series of experiments were performed with human papillomavirus (HPV) primers and HPV DNA-negative tissues and yielded equivalent results. Specifically, if the hot start PCR reaction conditions were employed where the *Taq* polymerase was not added until the block of the thermal cycler reached 55°C, then no signal was evident. However, when the *Taq* polymerase was added at room temperature at the onset of PCR, a 2+ to 3+ signal was evident in the skin and tonsil tissues (Fig. 5).

The final issue addressed was whether target-specific direct incorporation of digoxigenin dUTP into DNA could be accomplished in the tissue sections using the in situ PCR mechanism. This would presuppose conditions that would permit neither the primer-independent nor the nonspecific primer-dependent pathways. We used both frozen, fixed tissues and paraffin-embedded tissues successfully blocked with dideoxy-TTP pretreatment under hot start conditions. These experiments were done with vulvar warts and yielded equivalent results. These tissues contain HPV DNA that is present in the granular cell layer of the epithelium but not the basal epithelial layer or in cells in the dermis as described previously.[2,7–9] It was demonstrated that under hot start conditions the digoxigenin dUTP incorporated only in the granular layer cells when HPV-specific primers were employed with frozen, fixed tissue or paraffin-embedded tissue successfully blocked with the dideoxy-TTP treatment. Note that in the latter tissue the signal was seen in cells in the dermis if no dideoxy treatment was employed (Fig. 6), or if the dideoxy blockage was done under sub- or supraoptimal protease digestion (not shown), or if hot start was not employed after successful dideoxy blockage (not shown). Positivity in the dermis or in the basal epithelia layer must represent nonspecific signal.

DISCUSSION

This study analyzed the various pathways for DNA synthesis that could occur in the nucleus during in situ PCR as well as in solution-phase PCR. It was demonstrated that a major source of nonspecific DNA synthesis during in situ PCR was through a primer-independent mechanism. Prior heating of the tissue at 65°C was prerequisite for the manifestation of primer-independent DNA synthesis during in situ PCR. This pathway presumably reflects *Taq* polymerase-mediated repair of DNA gaps or nicks. Supporting this statement was the ability of a pretreatment DNA synthesis step, which included a dideoxynucleotide to block the primer-independent signal with in situ PCR. The requirement that the DNA be exposed to heat in the presence of a protein and a cross-linking fixative for the primer-independent signal was demonstrated for solution-phase PCR both with human DNA and highly purified salmon sperm DNA.

Elimination of the primer-independent and mispriming pathways by DNase digestion revealed that DNA synthesis secondary to primer oligomerization does not produce a detectable signal in the cell during in situ PCR. Furthermore, misprimed mediated DNA synthesis may occur in the nucleus during in situ PCR but could be eliminated by increasing the annealing temperature of

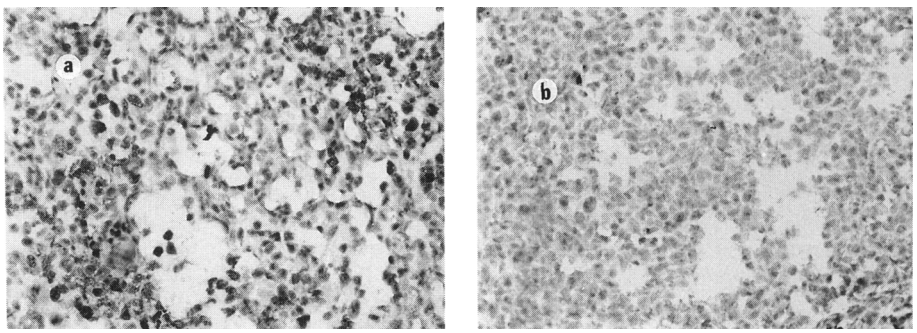

FIGURE 5 Effect of mispriming on the signal during in situ PCR. A signal was noted in this tonsil biopsy that was frozen, sectioned, and fixed in formalin during in situ PCR if nonspecific (HPV-specific) primers were used and the *Taq* polymerase was added at room temperature (*a*). The signal was lost if the *Taq* polymerase was not added until the reaction temperature reached 55°C (*b*).

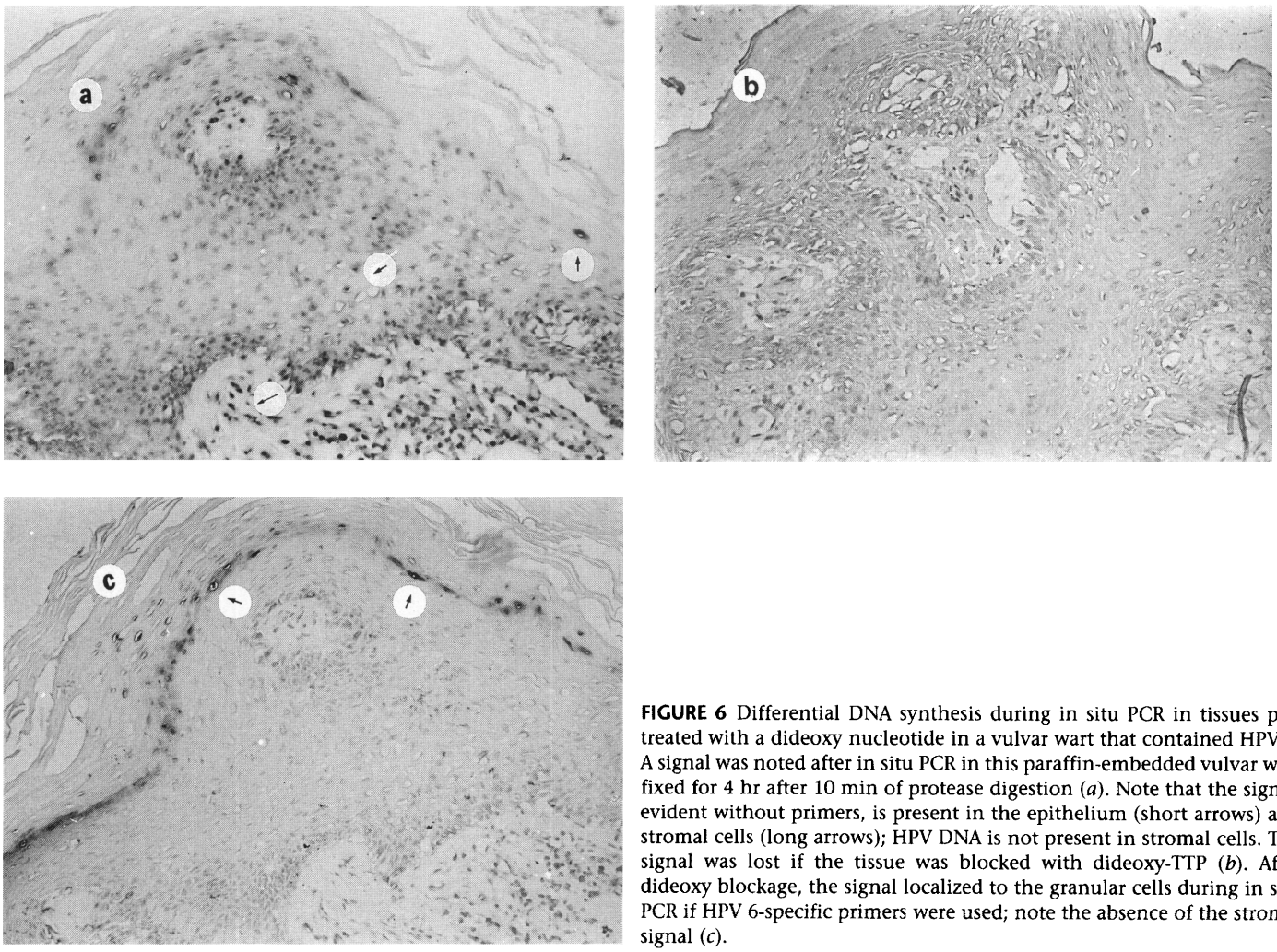

FIGURE 6 Differential DNA synthesis during in situ PCR in tissues pretreated with a dideoxy nucleotide in a vulvar wart that contained HPV 6. A signal was noted after in situ PCR in this paraffin-embedded vulvar wart fixed for 4 hr after 10 min of protease digestion (*a*). Note that the signal, evident without primers, is present in the epithelium (short arrows) and stromal cells (long arrows); HPV DNA is not present in stromal cells. The signal was lost if the tissue was blocked with dideoxy-TTP (*b*). After dideoxy blockage, the signal localized to the granular cells during in situ PCR if HPV 6-specific primers were used; note the absence of the stromal signal (*c*).

the PCR reaction above that of the primer–non-target melting through the hot start maneuver.

DNase activity in paraffin-embedded tissues that were suboptimally proteased enhanced the primer-independent signal with in situ PCR. Interestingly, this enhancement after suboptimal protease digestion was also seen in the dideoxy pretreatment reaction. In the latter case, the loss of the enhancement with substitution of the *Taq* polymerase by the Stoffel enzyme in the dideoxy-TTP pretreatment step suggests that the 5′ → 3′ exonuclease activity of the *Taq* polymerase may have been a factor in that enhancement. If one assumes that the primer-independent signal during in situ PCR represents repair of DNA gaps/nicks, this enhancement with suboptimal protease digestion and DNase digestion may represent enlargement of these gaps. This may be possible with suboptimal protease digestion, as the persisting protein–DNA cross-links prevents the total degradation of the DNA template by the DNase or complete blocking of the single-stranded gaps by dideoxy-TTP incorporation, respectively. The basis of the unexpected inability of the dideoxy-TTP pretreatment to block the primer-independent signal at supraoptimal protease conditions is not clear. This supraoptimal enhancement with dideoxy pretreatment is not related to the 5′ exonuclease activity of *Taq*, as it was also evident when the Stoffel enzyme was employed in the pretreatment step. If the enhancement reflects the exposure of regions of single-stranded DNA that exceed the amount that can be blocked with dideoxy-TTP pretreatment, it is feasible that increased time of dideoxy blockage could eliminate the signal seen with supraoptimal protease digestion. However, the signal was still evident under these conditions when the dideoxy-TTP pretreatment was increased from 30 to 60 min (G.J. Nuovo, unpubl.). Whether this reflects continual inability to block all potential sites of primer-independent DNA synthesis or some other mechanism requires further study.

PCR in situ hybridization differs from in situ PCR in that the reporter molecule is not directly incorporated during PCR. Rather, the PCR product in the cell is detected with an in situ hybridization step that uses a labeled internal oligoprobe. We have reported previously that the hot start maneuver increases the sensitivity of PCR in situ hybridization such that one can detect one target copy per cell using a single primer pair.[2,3] The hot start modification could either be done manually or by the use of uracil *N*-glycosylase or the single-stranded binding protein of *Escherichia coli*.[2,3] Similar enhancement of the sensitivity

of solution-phase PCR was reported with the manual hot start maneuver.[3,10,11] The observations in this study suggest that this enhancement in sensitivity during PCR in situ hybridization is attributable to inhibition of mispriming as the primer-independent mechanism is still operative under hot start conditions. It was also reported that target-specific in situ PCR could be done in cytospins under hot start conditions.[2,3] This study shows that this is possible because cytospin preparations are not routinely subjected to prolonged pretreatment at 65°C. When cytospin preparations of peripheral blood mononuclear cells were heated for 4 hr at 65°C, a primer-independent signal was seen after in situ PCR even with hot start conditions (G.J. Nuovo, unpubl.).

From the standpoint of one wishing to perform in situ PCR, several important points are evident from this study. First, one may detect RNAs (cDNAs) with direct incorporation (reverse transcription in situ PCR), as predigestion with RNase-free DNase eliminates the potential pathways for nonspecific DNA synthesis. However, to remove the potential for a false-positive result it is critical to determine that the protease digestion time is optimal for the length of time that the tissue was fixed. This optimal protease time is defined by achieving a 3+ signal with no DNase digestion and 0 signal after DNase digestion. Second, target-specific in situ PCR for DNA can be done on tissue sections under certain strictly defined conditions. One such condition is to use frozen tissue that is fixed after cryostat sectioning. At times, only fixed, paraffin-embedded tissue is available. Under these conditions, it is possible to obtain a target-specific signal with in situ PCR using a pretreatment with a dideoxynucleotide. However, it is important to stress that the range of optimal protease conditions for such tissues is narrow. Nonspecific DNA synthesis will be evident at both above and below the optimal protease digestion times. It is recommended that one use hot start PCR in situ hybridization, employing a labeled internal oligoprobe, when analyzing paraffin-embedded tissues for in situ PCR-amplified DNA targets. Additional studies are needed to discover other more reliable methods to eliminate the primer-independent DNA synthesis in paraffin embedded tissue sections during in situ PCR.

ACKNOWLEDGMENTS

We greatly appreciate many helpful discussions with John Atwood and Dr. Lawrence Haff from Perkin-Elmer Corporation who suggested the utility of the dideoxy-TTP pretreatment experiments. Dr. Eric Spitzer is thanked for critical comments, including his suggestion of the experiments with the purified salmon sperm DNA. Ms. Angella Forde, Michelle Margiotta, Kim Rhatigan, and Camille Kuschner provided excellent technical assistance. Ms. Sandra Otera provided expert photographic assistance. We are especially appreciative of Ms. Eileen Bolyston for her patience and skill in processing the tissues at different time points in paraffin. This work was supported by grants from Perkin-Elmer Corporation, Procter and Gamble Corporation, the Center for Biotechnology of the State of New York, and the Lewis Foundation (to G.J.N.).

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Received May 12, 1994; accepted in revised form July 25, 1994.