Constraints imposed by supercoiling on \textit{in vitro} amplification of polyomavirus DNA

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Previous attempts to identify oncogenic polyomaviruses in human cancers have yielded conflicting results, even with the application of PCR technology. Here, it was considered whether the topological features of the polyomavirus genome interfere with efficient PCR amplification. Plasmid and SV40 DNAs were used as a model system for comparing the amplification efficiency of supercoiled, circular relaxed and linear templates. It was found that detection of circular templates required 10 times more molecules than detection of identical but linear templates. Supercoiling hindered the \textit{in vitro} amplification of SV40 circles by a factor of 10, and erratic amplification of supercoiled SV40 occurred with subpicogram amounts of template. Accordingly, topoisomerase I treatment of DNA improved the PCR detection of supercoiled SV40, significantly decreasing the number of false-negative samples. Previously described, yet controversial, polyomavirus presence in human tissues should be reconsidered and topoisomerase I-sensitive polyomavirus amplification might help to detect polyomavirus genomes in mammalian tissues.

\textbf{INTRODUCTION}

The polyomavirus genome is a small, double-stranded, closed and supercoiled DNA molecule (Weil & Vinograd, 1963). Supercoiling confers very different physical properties on DNA from those of linear DNA molecules. Such properties allowed the identification and initial characterization of polyomavirus DNA, which was achieved by studying its sedimentation behaviour and by electron microscopy (Weil & Vinograd, 1963; Vinograd \textit{et al}., 1965). At the time of these initial studies, it was determined that heat- or alkali-induced denaturation of supercoiled DNA is seldom, if ever, achieved in the absence of strand breaking (Weil & Vinograd, 1963; Vinograd \textit{et al}., 1965, 1968). Later, confirmatory structural studies exploited the modifications induced by topoisomerase I, which unravels supercoiled genomes by introducing temporary breaks into one of the strands and thus removing the superhelical twists with a step-wise mechanism (Keller & Wendel, 1975). Recent attempts to analyse human tissues potentially harbouring polyomavirus genomes have not taken the properties of supercoiled DNA into account. Until the development of PCR, the search for polyomavirus genomes was based on hybridization studies. Since then, the use of PCR for detecting polyomavirus DNA in human tissues, particularly in tumour tissues, has generated discrepant results, not only among different studies but also within the same study (Carbone \textit{et al}., 1996, 1997; Testa \textit{et al}., 1998). The poor reproducibility of polyomavirus detection reported in human cancers has even suggested the possibility that false-positive samples were caused by contamination of amplification reactions (Pennisi, 1997). In our previous research, in which we used PCR to detect polyomavirus JC virus (JCV) DNA in a series of human colorectal cancers, we also experienced difficulties in reproducing results (Laghi \textit{et al}., 1999). We reasoned that potential limitations encountered in the detection of non-linear genomes with PCR had not been thoroughly considered, despite the fact that Mullis & Faloona (1987) had already described in their original PCR report that closed plasmids were not as efficiently amplified as mammalian DNA. Specifically, we focused on the hypothesis that a closed, supercoiled configuration of DNA might decrease the efficiency of the amplification reaction. Therefore, we introduced topoisomerase I treatment as a specific means of improving PCR-based detection of the JCV genome from frozen specimens of human colon cancers and matched normal colonic mucosa (Laghi \textit{et al}., 1999) and of normal human foregut (Ricciardiello \textit{et al}., 2000).

Here, we systematically investigated the hypothesis that the efficiency and reproducibility of \textit{in vitro} amplification of a DNA sequence is dependent on its topology. To test this hypothesis, we assessed whether DNA with a closed
conformation was amplified in vitro as efficiently as linear DNA and whether supercoiling further affected amplification efficiency. Thereafter, we compared the efficiency of in vitro amplification of a supercoiled genome with traditional PCR and with PCR combined with topoisomerase I treatment.

METHODS

Templates and enzymes. Plasmid pZut was constructed by subcloning an ampiclon that included the JCV origin of replication and the adjacent T/t antigen 5′-coding portion into pUC18. The reference strain SV40T strain DNA, restriction enzymes and bovine topoisomerase I were purchased from Gibco Life Science, while yeast topoisomerase I was purchased from Epicentre (yeast topoisomerase I is no longer commercially available). All enzymic reactions were performed according to the manufacturer’s instructions. The colon cancer cell lines SW480, HCT116, LoVo, HT29 and HeLa cells were obtained from ATCC and grown under recommended conditions. DNA was extracted after proteinase K digestion using standard phenol/chloroform purification and ethanol precipitation.

Fractionation and recovery of individual DNA topologies. Enzyme-treated and untreated samples were electrophoresed through 1-75% SeaPlaque GTG agarose gels (FMC). After electrophoresis, gels were post-stained with ethidium bromide and bands corresponding to individual conformations were excised. DNA was recovered from agarose with agarase, which was used according to the manufacturer’s instructions, and without the use of phenol. The A_{260} of the recovered DNA was used to prepare 10 ng ml^{-1} stock solutions for 10-fold dilution of templates.

PCR. Amplification reactions were carried out in 50 or 100 µl with reagents purchased from Gibco in a thermal cycler (Hybaid). Each vial contained the indicated amount of specific template plus the background provided by 1 µg human Cot DNA. The amplification mix contained 20 mM Tris/HCl (pH 8.4), 50 mM KC1, 100 mM dNTPs, 10 pmol each primer and 1-25 U Taq polymerase. To amplify the 5′-coding portion of JCV T/t antigen from pZut, samples were denatured in the presence of 1-5 mM MgCl2 at 94°C for 1 min, followed by 27 cycles of 92°C for 1 min, 50°C for 30 s and 72°C for 45 s with the following primers: 5′-AATAGTGGA-TTTACCTTAAAG-3′ (forward) and 5′-TGATAGGAGGAATCC-TGCGG-3′ (reverse). The same primers and conditions were used for a second round. Southern blotting of amplification products was performed using 1 % of the first reaction product as template for the second round. Fractionation of individual DNA topologies. Enzyme-treated and untreated samples were electrophoresed through 1-75% SeaPlaque GTG agarose gels (FMC). After electrophoresis, gels were post-stained with ethidium bromide and bands corresponding to individual conformations were excised. DNA was recovered from agarose with agarase, which was used according to the manufacturer’s instructions, and without the use of phenol. The A_{260} of the recovered DNA was used to prepare 10 ng ml^{-1} stock solutions for 10-fold dilution of templates.

RESULTS

We first compared the in vitro amplification of an identical template sequence in its linear and closed conformations. For this experiment, we used the native and EcoRI-linearized forms of the pZut plasmid, selectively recovered after agarose gel electrophoresis (Fig. 1a). We used 10-fold serial dilutions of equimolar amounts of each conformation for PCR. We found that the linearized plasmid could be detected by PCR at a 10-fold lower dilution than the closed plasmid (Fig. 1b).

Next, to investigate whether supercoiling imposes a further restriction on the in vitro amplification of circular DNA molecules, we used an SV40 DNA model to compare the amplification of two different closed conformations. Agarose gel electrophoresis was used to fractionate the

![Fig. 1. Constraints imposed by a closed topology on in vitro amplification of DNA. (a) Gel separation prior to the recovery of individual DNA conformations of the pZut plasmid, showing the native, closed form (lane 1) and EcoRI-linearized form (lane 3) of pZut. More than one DNA conformation per lane was detectable after separation. Lane 2, molecular mass marker. (b) Equimolar amounts of linear and closed pZut are not equally amplified. PCR products from paired, 10-fold dilutions (10 ng, 1 ng, 100 pg and 10 pg) of closed (lanes 1–4, respectively) and EcoRI-linearized (lanes 7–10, respectively) pZut obtained after 27 cycles of PCR. Lane 6, molecular mass marker; lanes 5 and 11, negative controls with no plasmid DNA.](image-url)
native, supercoiled (form I, with 20–24 supercoils) and the circular, relaxed (form II, with zero supercoils) SV40 DNA obtained by treating the viral genome with bovine topoisomerase I (Fig. 2a). Again, equimolar 10-fold dilutions of the two conformations were not equally amplified (Fig. 2b). On the basis of these results, we studied the sensitivity of PCR for specific DNA conformations near the limit of detection, the end-point dilution. These experiments showed that 10 fg SV40 DNA was sufficient to detect the circular but not the supercoiled form (Fig. 2c and d).

After determining the different end-point dilutions for the individual topologies of closed DNA, we investigated whether combining the two reactions – the unravelling of supercoiled DNA and in vitro amplification – would improve the sensitivity of detection of a supercoiled genome. Towards this aim, we compared the combination of topoisomerase I treatment and PCR with traditional PCR alone for the detection of subpicogram amounts of supercoiled SV40. To avoid introducing a buffer condition that might inhibit the amplification reactions, we first tested the topoisomerase I activity in PCR buffers with modified ionic strengths (Fig. 3a); salt re-equilibration achieved by volume adjustments after topoisomerase I treatment was sufficient to restore a standard PCR buffer for optimal Taq polymerase efficiency. For the 20 combined topoisomerase I and PCR samples, detection of SV40 DNA yielded 18 positive and two false-negative reactions. In contrast, for the 20 PCR-only reactions, detection of SV40 yielded seven positive and 13 false-negative reactions (Table 1; Fig. 3b). As observed in reconstitution experiments, TISPA also proved to be more sensitive than PCR alone in the detection of supercoiled viral DNA from human cancer cells. We have reported the presence of JCV DNA in the human colon cancer cell line SW480 (Laghi et al., 1999), but not in other colon cancer cell lines. By using TISPA to confirm our previous findings (Fig. 4a), we also showed by a titration experiment that TISPA could detect the JCV genome from one quarter of the amount of SW480 DNA required for the less-sensitive detection by PCR alone (Fig. 4b).

**DISCUSSION**

Because our previous attempts to use PCR to detect the supercoiled JCV genome in human normal colon and colon cancer samples yielded inconsistent results, we initiated the use of topoisomerase I in conjunction with PCR (Laghi et al., 1999; Ricciardiello et al., 2000). Here, we extended the scope of our previous experiments by analytically investigating the efficiency of in vitro amplification of small
linear and non-linear DNA molecules and by documenting the different sensitivities of amplification that were achieved from combined topoisomerase I treatment and PCR versus PCR alone.

Pre-PCR enzymic digestion has been used to achieve amplification of whole-length supercoiled genomes (Agostini & Stoner, 1995; Lednicky et al., 1997) but only semi-quantitative data are available. Furthermore, in our preliminary experiments, we found that the SspI and EcoRI buffers used to linearize closed DNA molecules inhibited Taq polymerase (data not shown). Thus, we developed a model to avoid both difficult digests of supercoiled DNA (Snounou & Malcom, 1984) and the potential inhibition of Taq polymerase activity by enzyme digests. In fact, due to the identical processing of the templates with different conformations, PCR inhibition could not account for the decreased efficiency of amplification reactions of the non-linear DNA conformations. Rather, we found both closed topology and supercoiling to decrease the amplification efficiency of given DNA sequences. As the two closed conformations of SV40 differed with respect to their endpoint dilution, we inferred that DNA supercoiling further hindered the amplification of the closed circular template. Accordingly, the removal of supercoils from closed circles prior to PCR resulted in an increased detection rate, mainly near the limit of detection. As the same preparation of the amplification of whole-length supercoiled genomes (Agostini & Stoner, 1995; Lednicky et al., 1997) but only semi-quantitative data are available.

**Table 1. Detection of subpicogram quantities of supercoiled SV40<sub>776</sub> against a background of 1 µg human DNA: comparison of traditional PCR versus TISPA**

<table>
<thead>
<tr>
<th>Template molecules</th>
<th>PCR-positive</th>
<th>TISPA-positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>Amount (fg)</td>
<td></td>
</tr>
<tr>
<td>1600*</td>
<td>10</td>
<td>0/5</td>
</tr>
<tr>
<td>1600†</td>
<td>10</td>
<td>3/5</td>
</tr>
<tr>
<td>800†</td>
<td>5</td>
<td>4/10</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>7/20‡</td>
</tr>
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</table>

*Experiments performed using a 40-cycle amplification.
†Experiments performed with nested amplification, using 35 cycles for the first round and 26 cycles for the second.
‡P<0.001 using Fisher’s exact test to compare the number of negative samples in TISPA and PCR experiments.
agarose-recovered supercoiled SV40 DNA was used in the amplification reactions with and without topoisomerase I treatment, these results provide further evidence that supercoiling hinders the amplification efficiency of the same but enzymatically relaxed circular DNA. Of relevance, the relaxing reactions were performed in a slightly modified PCR buffer, yet appropriate for both topoisomerase I and Taq polymerase.

Unique features of supercoiled polyomavirus DNA need to be considered with regard to the theoretical basis of the in vitro amplification process. First, the denaturation of supercoiled molecules requires temperatures exceeding 100 °C (Vinograd et al., 1965, 1968). Second, supercoiled DNA may still not be accessible to the primers once heat-denatured. In fact, denaturation of supercoiled DNA without concurrent strand nicking results in a compact DNA shape, the fast-sedimenting double-stranded cyclic coil (Keller & Wendel, 1975), rather than in two separate strands. Although strand nicking is expected during thermal cycling because of heat-induced depurination, this is unpredictable, and the resulting linear molecules will be exponentially amplified only if they are present in the initial cycles. Complicating matters further, the recovery of polyomavirus genomes during DNA extraction is linked both to the extent of damage of the supercoiled molecules and to the sedimentation coefficient of each topological form (Vinograd et al., 1968). Notably, recovery might not reflect the actual viral load in mammalian tissues (Carbone et al., 1997; Hirt, 1967). Thus, both the final number and the specific conformation of polyomavirus genomes may greatly vary in DNA preparations. Consequently, variable proportions of linear polyomavirus DNA (form III) in samples to be tested could account for the poor PCR reproducibility (Carbone et al., 1997; Laghi et al., 1999). In PCR conditions that would readily detect a linear sequence, if the pool of polyomavirus DNA molecules to be amplified is small (i.e. ≤1 pg in our experimental conditions), then very few, if any, linearized molecules might be available for primer annealing or be suitable for elongation. This issue, unlikely to be relevant in the presence of a lytic infection (White et al., 1992; Tornatore et al., 1992), needs to be considered in the absence of active virus replication. Our data from the cancer cell line SW480 also points in this direction, as traditional PCR required higher amounts of DNA than TISPA in order to detect the supercoiled polyomavirus genome. Accordingly, TISPA might improve the yield of positive samples, when looking for polyomavirus genomes in mammalian cells not prone to lytic infection.

Our data document the different PCR efficiencies for each topological form of polyomavirus DNA. Consequently, we propose that PCR-based detection of any supercoiled DNA exploits the amplification of a fraction of linearized molecules that randomly originate during nucleic acid extraction and manipulations or even during thermal cycling. We have shown that topoisomerase I treatment can increase the detection rate of a supercoiled genome near its endpoint dilution for a given PCR condition, an approach that should allow increased consistency in the identification of human tissues harbouring polyomaviruses (Laghi et al., 1999; Ricciardiello et al., 2000). Understanding the current limitations of the in vitro amplification of supercoiled genomes and improving the recognition of polyomavirus genomes might provide new potential targets for involvement in the molecular pathogenesis of human diseases, including cancer.

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