Distinct patterns of alteration of myc genes associated with integration of human papillomavirus type 16 or type 45 DNA in two genital tumours

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We previously described two genital carcinomas (IC2, IC4) containing human papillomavirus type 16 (HPV-16)- or HPV-18-related sequences integrated in chromosomal bands containing the c-myc (8q24) or N-myc (2p24) gene, respectively. The c-myc gene was rearranged and amplified in IC2 cells without evidence of overexpression. The N-myc gene was amplified and highly transcribed in IC4 cells. Here, the sequence of an 8039 bp IC4 DNA fragment containing the integrated viral sequences and the cellular junctions is reported. A 3948 bp segment of the genome of HPV-45 encompassing the upstream regulatory region and the E6 and E7 ORFs was integrated into the untranslated part of N-myc exon 3, upstream of the N-myc polyadenylation signal. Both N-myc and HPV-45 sequences were amplified 10- to 20-fold. The 3’ ends of the major N-myc transcript were mapped upstream of the 5’ junction. A minor N-myc/HPV-45 fusion transcript was also identified, as well as two abundant transcripts from the HPV-45 E6–E7 region. Large amounts of N-myc protein were detected in IC4 cells. A major alteration of c-myc sequences in IC2 cells involved the insertion of a non-coding sequence into the second intron and their co-amplification with the third exon, without any evidence for the integration of HPV-16 sequences within or close to the gene. Different patterns of myc gene alterations may thus be associated with integration of HPV DNA in genital tumours, including the activation of the protooncogene via a mechanism of insertional mutagenesis and/or gene amplification.

Introduction

Specific types of human papillomavirus (HPV) are associated with the development of genital neoplasia (zur Hausen, 1996). The integration of viral sequences into the cell genome has been reported in intraepithelial cancer precursors (Cullen et al., 1991; Daniel et al., 1997; Schneider-Maunoury et al., 1987) and is frequently observed in invasive carcinomas (Cullen et al., 1991; Dürst et al., 1985; Shah & Howley, 1996). It is likely to constitute a crucial event in tumour progression. By inactivating the regulatory viral E2 gene, integration results in the constitutive expression of the viral E6 and E7 oncoproteins, which should favour the emergence of immortalized keratinocyte clones and generate genomic instability (Jeon & Lambert, 1995; Romanczuk & Howley, 1992; Schwarz et al., 1985; Shah & Howley, 1996). Integration could also play a role by activating or inactivating cellular genes involved in the control of keratinocyte growth and differentiation (Couturier et al., 1991; Reuter et al., 1998), but such a mechanism is still poorly documented. Recurrent integration of HPV DNA sequences in chromosomal regions containing myc genes has been observed in cases of invasive genital carcinomas and in cervical cancer-derived cell lines (Couturier et al., 1991; Dürst et al., 1987; Hori et al., 1991; Lazo et al., 1992; Macville et al., 1999; Popescu et al., 1990). It is worth stressing that the c-myc gene has been reported to be often rearranged and/or amplified in invasive cervical carcinomas (Ocadiz et al., 1987; Riou et al., 1985) and the up-regulation of c-myc expression has been found to be a prognostic factor for tumour progression (Bourhis et al., 1990).

In order to investigate the relationships between integration of HPV sequences and alterations of the structure and expression of a myc protooncogene, we have further analysed two genital invasive carcinomas (IC2 and IC4) previously found to harbour HPV-16- and HPV-18-related sequences.
respectively, integrated at the chromosomal sites of c-myc (IC2) and N-myc (IC4) (Couturier et al., 1991). The IC2 tumour had been found to harbour rearranged and amplified c-myc sequences without any evidence of c-myc overexpression, whereas a 10- to 20-fold amplification of the N-myc gene and high levels of N-myc transcripts had been observed in the IC4 tumour (Couturier et al., 1991).

Our data show that DNA sequences of HPV-45 are integrated in the 3’ untranslated part of the N-myc gene in IC4 cells, that a major N-myc transcript terminates upstream of the virus–cell junction and that high levels of N-myc protein are expressed. In contrast, we found no evidence for integration of HPV-16 within or in the vicinity of the c-myc gene in IC2 cells and we show that the major c-myc rearrangement corresponds to the insertion of a non-coding cellular sequence into the second intron of the gene.

**Methods**

- **Tumours and tumour-derived cell lines.** The genital tumours analysed corresponded to a well-differentiated invasive squamous cell carcinoma (SCC) of the penis (IC2) and to a poorly differentiated SCC of the uterine cervix (IC4) of French Caucasian patients. Biopsy specimens were taken before treatment. A part of each biopsy was fixed in formalin for histological analysis and the remaining neoplastic tissue was frozen in liquid nitrogen and stored at -80°C. IC2 and IC4 tumour-derived cell lines were grown from explants of tumour fragments on collagen-coated flasks, as described previously (Couturier et al., 1991). SKNBE cells derived from a human neuroblastoma (Biedler & Spengler, 1976), and found to contain about 50 copies of N-myc (de Crémoix et al., 1997), were grown in RPMI medium supplemented with 10% foetal calf serum.

- **Dual-colour-fluorescent in situ hybridization.** Chromosome preparations of IC2 and IC4 cells obtained at the 12th subculture were hybridized with a mixture of probes specific for HPV-16 and c-myc (IC2) or for HPV-45 and N-myc (IC4). HPV DNA probes were biotin-labelled by using a nick translation kit (Boehringer Mannheim). Digoxigenin-labelled c-myc and N-myc DNA probes were purchased from Oncor. After incubation in 2 × SSC at 37°C for 30 min and denaturation in 70% formamide/2 × SSC at 70°C for 2 min, chromosome preparations were hybridized with the denatured probes (5 ng/µl) in 50% formamide/2 × SSC at 37°C for 15 h. Slides were washed at 37°C in 50% formamide/2 × SSC and then 2 × SSC. Detection was achieved by using an FITC-avidin/anti-digoxigenin rhodamine solution (Oncor). Slides were mounted in Vectashield-DAPI (Vector Laboratories). Fluorescence observation and image capture were performed on a Leica DMRB microscope fitted with a Quanix digital camera (Photometrics). The three-colour images (DAPI, FITC and rhodamine) were processed on a Quips Smart Capture workstation (Vysis).

- **DNA, RNA and protein preparation.** Total DNA was prepared from tumour biopsy specimens and human placenta by standard methods of phenol–chloroform extraction followed by ethanol precipitation (Sambrook et al., 1989). DNA, RNA and proteins were extracted simultaneously as described by Coombs et al. (1990). Briefly, frozen samples corresponding to 104 IC2 cells (12th subculture) and tumours (100 mg) obtained after grafting 105 IC4 cells (12th subculture) or 106 SKNBE cells to athymic mice were homogenized in 4 M guanidinium thiocyanate by using an Ultraturrax T25 homogenizer (Janke & Kunkel, IKA-Labortechnik). The homogenates were layered onto a 5 M caesium chloride cushion and centrifuged for 18 h at 130 000 g at 20°C.

RNA was recovered from the pellet by dissolution in 0.3 M sodium acetate (pH 6) and ethanol precipitation. DNA was extracted by phenol–chloroform and ethanol precipitation from the caesium chloride layer after dialysis and incubation for 24 h with proteinase K (10 µg/ml). The protein-containing guanidine phase was dialysed against 100 mM ammonium bicarbonate and lyophilized. DNA and RNA concentrations were measured by spectrophotometry at 260 nm and proteins were quantified by using the Bio-Rad DC protein assay.

- **Southern blot hybridization experiments.** Total cell DNA preparations (10 µg) obtained from tumour biopsy specimens and from human placenta were digested with BamHI, EcoRI, HindIII or PstI restriction endonucleases, electrophoresed in 0.8% agarose gels, blotted onto nitrocellulose filters (Hybond-C, Amersham International) and hybridized with random-primed, 32P-labelled DNA probes (Couturier et al., 1991). Probes corresponded to cloned HPV-16 and HPV-45 genomes excised from plasmid sequences, as well as to PCR amplification products obtained by using primers specific for c-myc exon 1 (nt 306–325 and 884–865), exon 2 (nt 4728–4747 and 5066–5047) and exon 3 (nt 6731–6750 and 7777–7758) (Gazin et al., 1984) and from the N-myc exon 3 (nt 5003–5022 and 5612–5593) (Stanton et al., 1986). Membranes were exposed to X-ray films for 16–48 h.

- **Molecular cloning of rearranged myc sequences.** DNA libraries were constructed by inserting EcoRI-digested IC4 tumour DNA in a λ gt ves vector (Life Technologies) or BamHI-treated IC2 tumour DNA in a λ GEM-11 vector (Promega). About 106 plaques were screened successively with N-myc exon 3 and HPV-45 DNA probes (IC4) or with c-myc exon 3 and HPV-16 DNA probes (IC2). Recombinant λ phages were isolated as described previously (Sambrook et al., 1989) from three plates that hybridized with both N-myc and HPV-45 probes and were found to contain a 15 kb DNA fragment. An EcoRI–Xhol fragment of 8 kb (IC4-EX-8kb) containing both N-myc and HPV-45 sequences was subcloned in pBluescript (Stratagene). Screening of the IC2 library yielded six plaques that hybridized with c-myc, five with HPV-16 and none with both probes. The c-myc-positive plaques were found to contain recombinant λ phages with an 8 kb insert. A BamHI–EcoRI DNA fragment of 3 kb (IC2-BE-3kb) containing the c-myc sequences was subcloned in pBluescript plasmid.

- **Nucleotide sequence analysis.** The nucleotide sequences of the cloned IC4-EX-8kb and IC2-BE-3kb DNA fragments were determined by using the ABI prism Bigdyne terminator cycle sequencing kit with AmpliTaq FS DNA polymerase (Perkin-Elmer) as recommended by the supplier. Primers used corresponded to M13 oligonucleotide sequences complementary to sequences flanking the inserted fragment (M13 universal and reverse primers) and to internal oligonucleotides. Internal primers in both orientations were specific for N-myc intron 2 and exon 3 sequences (nt 3718–3737, 4211–4230, 4761–4780, 5265–5284, 5781–5800 and 6214–6233) (Stanton et al., 1986), HPV-45 sequences (nt 5632–5651, 6140–6169, 6069–6068, 7114–7133, 7573–7592, 264–283, 574–593 and 1096–1115) (Myers et al., 1994) and c-myc intron 2 and exon 3 sequences (nt 5723–5742, 6269–6288, 6731–6750, 7192–7211 and 7557–7576) (Gazin et al., 1984). Sequencing products were analysed with an ABI prism 377 DNA sequencer (Perkin-Elmer). Multiple alignments of DNA sequences were performed by using the CLUSTAL W program.

- **Northern blot hybridization experiments.** Samples (10 µg) of total RNA obtained from IC4 cells were electrophoresed in a denaturing (6% formaldehyde) 1% agarose gel and blotted onto a nitrocellulose membrane (Hybond-C, Amersham International) and strips were hybridized in parallel with different 32P-labelled DNA probes. Probes were prepared from genomic HPV-45 DNA or PCR amplification.
products obtained by using primers specific for N-myc exon 3 (nt 5003–5022 and 5612–5593) (Stanton et al., 1986), HPV-45 upstream regulatory region (URR) (nt 7271–7289 and 7705–7686) and HPV-45 ORFs E6 (nt 63–82 and 593–574), E7 (nt 574–593 and 920–901) and L1 (nt 5209–5228 and 7007–6988) (Myers et al., 1994). Membranes were exposed to X-ray films for 10–48 h.

**Mapping of the 3′ ends of N-myc transcripts in IC4 cells.** Total RNA (1 µg) isolated from IC4 cells was reverse-transcribed with the Superscript RT RNase H reverse transcription kit (Life Technologies), in the presence of an antisense adaptor primer [5′ GACTCGAGTCGA-CCCGGG(dT)12 3′], according to the instructions of the manufacturer. After ethanol precipitation and centrifugation, the pellet was suspended in distilled water (20 µl) and 2 µl of the cDNA mixture was used for PCR amplification (Frohman et al., 1988). Primers were specific for the untranslated part of N-myc exon 3 (nt 6077–6096) (Stanton et al., 1986) and for the adaptor. PCR products were cloned in the Smal site of pBluescript (Stratagene) and sequenced by using M13 universal and reverse primers.

**Detection of N-myc protein.** Aliquots of protein extracts (50 µg) and prestained protein markers (New England BioLabs) were electrophoresed on a 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schuell). The membrane was incubated with sheep anti-human N-myc IgG (Serotec) at a dilution of 1:200. After washing, the membrane was incubated with peroxidase-conjugated rabbit antibodies raised against sheep IgG (Dako) at a dilution of 1:1000. Proteins were visualized with the ECL chemiluminescent detection system as described by the manufacturer (Amersham International).

**Results**

**Co-localization of HPV and myc sequences in IC2 and IC4 cells**

Sequencing of the region of the L1 ORF amplified from IC4 DNA by using the MY11/MY09 consensus primers (Manos et al., 1989) showed that the integrated HPV-18-related sequences harbouring the IC4 carcinoma (Couturier et al., 1991) corresponded to the Is040 HPV-45 variant (Stewart et al., 1996). Chromosome preparations of peritetraploid IC2 cells and pseudodiploid IC4 cells were hybridized with DNA probes specific for HPV-16 and c-myc and for HPV-45 and N-myc, respectively. Superimposition of the signals corresponding to each probe was observed on one of the chromosomes 8 for IC2 cells (Fig. 1A) and on the 2q+ chromosome described previously for IC4 cells (Couturier et al., 1991) (Fig. 1B). The c-myc and N-myc signals were more intense than those observed on the other chromosomes 8 and on the normal chromosome 2, which harboured no viral sequences. These data strongly suggested a link between the amplification of myc genes and the integration of HPV sequences and warranted further analysis of the relationships between viral sequences and the protooncogenes.

**Integration of HPV-45 within N-myc exon 3 in the IC4 tumour**

Previous analysis of IC4 DNA with an N-myc exon 2 probe disclosed a 10- to 15-fold amplification of the N-myc gene with no evidence for sequence rearrangement. Because an abnormal minor 5-6 kb N-myc transcript was detected (Couturier et al., 1991), we analysed the tumour DNA further with N-myc exon 1 and 3 probes. After cleavage with EcoRI, an enzyme that does not cut the N-myc gene (Stanton et al., 1986) or HPV-45 DNA (Myers et al., 1994), an oversized 15 kb fragment was detected with the N-myc exon 3 probe only, in addition to the normal 4 kb allele (Fig. 2A, lanes 2 and 3). Hybridization with an HPV-45 probe detected only the 15 kb fragment, with a signal intensity corresponding to about 10 copies of the viral genome (Fig. 2A, lane 1). This indicated that HPV sequences were integrated close to the third exon of the N-myc gene and that viral and cellular sequences were co-amplified.

The rearranged 15 kb EcoRI fragment was cloned in a bacteriophage λ vector and an 8 kb EcoRI–XhoI fragment (IC4-EX-8kb) that hybridized with both N-myc and HPV-45 probes was subcloned and sequenced. This revealed that the fragment (8039 bp) contained N-myc intron 2 and exon 3 sequences (3295 bp), HPV-45 sequences (3948 bp) extending from the 3′ end of the L2 ORF to the 5′ part of the E1 ORF and sequences (796 bp) that showed no relatedness to any of the sequences available in the EMBL database (Fig. 3A). Integration interrupted the N-myc gene at nucleotide T6518 or A6519, upstream of the AATAAA polyadenylation signal (nucleotide 6535) and the TGA stop codon (nucleotide 6556) (Stanton et al., 1986), and disrupted the HPV-45 L2 ORF at nucleotide T5117 or A5118 (Myers et al., 1994) (Fig. 3B). The 3′ virus–host junction was mapped to nucleotide 1217 in the HPV-45 E1 ORF (Fig. 3B). A fusion ORF was generated that encoded a putative protein that comprised the 120 N-terminal E1 amino acids and 33 residues of cellular origin (Fig. 3B).

No nucleotide changes were observed in the translated part of N-myc exon 3. Eight changes (mostly nucleotide insertions or deletions) were found in the N-myc intron 2 sequence and a single nucleotide change in the untranslated part of exon 3 (Table 1). In comparison to the prototypical HPV-45 sequence (Myers et al., 1994; Naghashfar et al., 1987), 48 nucleotide changes (1-2%) were observed in the integrated HPV-45 sequence, together with a nine-nucleotide in-frame deletion in the L1 ORF. Sixteen non-synonymous mutations led to five amino acid changes in the C-terminal end of the L2 protein, four amino acid changes in the L1 protein in addition to the three amino acid deletion, two amino acid changes in the E6 and E7 proteins and three amino acid changes in the N-terminal part of the E1 protein (Table 1).

**Two abnormal N-myc transcripts in the IC4 tumour cells**

No primary IC4 tumour specimen was available for RNA studies. An abundant 3-1 kb N-myc transcript with a size similar to that of the normal N-myc mRNA (Schwab et al., 1984) and a minor abnormal 5-6 kb transcript were found to be expressed in a nude mouse tumour obtained after grafting IC4 cells at the 6th subculture (Couturier et al., 1991). Because N-
myc amplification was present both in the primary tumour and in grafted tumour cells (Couturier et al., 1991), it was unlikely that the abnormal expression of N-myc was due to culture in vitro. Northern blot hybridization of total RNA prepared from grafted IC4 cells with an N-myc exon 3 probe or an HPV-45 probe showed that only the minor 5-6 kb species hybridized with both probes, thus corresponding to a fusion N-myc/HPV-45 transcript (Fig. 2B, lanes 1 and 2). In addition, two HPV-45 transcripts of 1.5 and 4.2 kb were detected with genomic or subgenomic E6 and E7 HPV-45 probes (Fig. 2B, lanes 2-4).

In order to map the 3′ ends of the N-myc transcripts, poly(A)^+ mRNAs of IC4 cells were reverse transcribed and the amplification products (about 200 bp) of the N-myc cDNA 3′ ends were cloned and sequenced. Three polyadenylation sites were identified, at positions 6208, 6290 and 6293 of the N-myc sequence (Fig. 4); i.e. upstream of the bona fide AATAAA N-myc polyadenylation signal that is deleted upon integration of HPV-45 DNA sequences. This makes it likely that the cryptic polyadenylation signals AATAATA (nucleotide position 6149) and AACTAAA (nucleotide position 6264), located in the untranslated part of N-myc exon 3, were used for the 3′-end processing of the major 3′-1 kb transcript. No cDNA corresponding to the minor 5-6 kb species was obtained. This transcript was found to hybridize with HPV-45 L1 and URR probes but not with the E6 and E7 probes (Fig. 2B, lanes 3–6). It is thus likely that one of the putative polyadenylation signals (AATAAA) located at nucleotide positions 7310 and 7671 in the HPV-45 URR (Myers et al., 1994) was used, resulting in the addition of about 2-2 or 2-5 kb of viral sequences to the 3′-1 kb N-myc transcripts. A summary of the transcription data is given in Fig. 3(A).

**N-myc protein overexpression in IC4 tumour cells**

In human tumour cell lines expressing amplified N-myc sequences, the N-myc protein is detected as two doublets of closely migrating phosphorylated (p62 and p64) and non-phosphorylated (p58 and p60) polypeptides that are translated from two in-frame AUG codons located 25 bp apart in the second exon of the N-myc gene (Makela et al., 1989). In order to determine whether high levels of N-myc mRNA in IC4 cells were associated with N-myc protein expression, Western blot
Fig. 3. Integration pattern of HPV-45 DNA sequences within N-myc in IC4 cells. The nucleotide sequence of a cloned EcoRI–XbaI DNA fragment containing the host–virus junctions (8039 bp) was aligned with the nucleotide sequences of the N-myc gene (Stanton et al., 1986) and the HPV-45 genome (Myers et al., 1994). (A) A schematic representation of the sequenced region is presented and includes intron 2 and exon 3 sequences of N-myc (nt 3231–6519), HPV-45 sequences (nt 5118–1217) and cellular DNA sequences of unknown origin (nt 1–796). The translated part of N-myc exon 3 is hatched and the in-frame ORF contained in the unknown sequences is represented by a black box. A genetic map of the integrated HPV-45 sequences is shown below and a representation of the N-myc exon 3 sequences deleted upon integration, which contain the AATAAA polyadenylation site, is shown above. The N-myc, N-myc/HPV-45 and HPV-45 E6–E7 transcripts detected in IC4 cells (see Figs 2B and 4) are represented at the bottom. The 3′ ends of the N-myc 3·1 kb mRNA and the sizes of the transcripts are indicated. (B) Nucleotide sequence of the 5′ and 3′ host–virus junctions. The 5′ host–virus junction is mapped to a TA dinucleotide located at positions 6518 and 6519 in the N-myc gene and 5117 and 5118 in the HPV-45 genome. The N-myc AATAAA polyadenylation signal and the TGA stop codon deleted upon integration are boxed and underlined, respectively. The 3′ junction is mapped to position 1217 in the HPV-45 DNA. The C-terminal amino acid sequence of a putative HPV-45 E1–cell fusion protein is given with the 33 residues of unknown origin in italics.
Table 1. Nucleotide and amino acid changes in the IC4-EX-8kb fragment

Insertions and deletions are indicated by + and −, respectively.

<table>
<thead>
<tr>
<th>Location</th>
<th>Nucleotide (amino acid) changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-myc</td>
<td></td>
</tr>
<tr>
<td>Intron 2</td>
<td>+GT at nt 3260, +C at nt 3502, −C4427, −A1500, +A at nt 4505, A1644 → T, +C at nt 4800, +GA at nt 4876 +G at nt 5847</td>
</tr>
<tr>
<td>Exon 3</td>
<td></td>
</tr>
<tr>
<td>untranslated part</td>
<td></td>
</tr>
<tr>
<td>HPV-45</td>
<td></td>
</tr>
<tr>
<td>L2 ORF 3’ end</td>
<td>A5247 → C (Ile183 → Leu), A5259 → G (Asn192 → Asp), C5291 → T, C5328 → A (His585 → Asn), C5357 → A, T5375 → C, T5383 → G (Ser151 → Ala), A5383 → T (Thr156 → Ser)</td>
</tr>
<tr>
<td>L1 ORF*</td>
<td></td>
</tr>
<tr>
<td>C1771 → C, A1771 → G, A1821 → G, G1859 → A, A1951 → C (Ile639 → Thr), C4677 → A (Ser346 → Asn), G4687 → T, C5685 → C (Gln195 → His), A2638 → G, C5857 → A, A5861 → A, deletion of nt 7090–7098 (Ser235, Thr236, Ala237), G7143 → A</td>
<td></td>
</tr>
<tr>
<td>URR</td>
<td></td>
</tr>
<tr>
<td>E6 ORF</td>
<td>A1241 → C (lys8 → Thr), T1560 → C, G2259 → T (Cys83 → Phe), T1842 → C</td>
</tr>
<tr>
<td>E7 ORF</td>
<td>C601 → A, A603 → C (Glu6 → Ala), A718 → C, G822 → T (Glu82 → Asp)</td>
</tr>
<tr>
<td>E1 ORF 5’ end</td>
<td>C967 → T, T9030 → C, C1124 → G (His71 → Asp), C1160 → G (His83 → Asp), C1177 → C (Lys88 → Asn)</td>
</tr>
</tbody>
</table>

* Amino acids are numbered from the first methionine.
N-myc protein from IC4 cells migrated slightly ahead of that from SKNBE cells. No N-myc protein was found in IC2 cells (Fig. 5).

Lack of evidence for integration of HPV-16 within c-myc in the IC2 tumour

Amplified rearranged c-myc sequences were detected previously with a c-myc exon 3 probe in IC2 cells, without any evidence of c-myc overexpression or an abnormal c-myc transcript (Couturier et al., 1991). IC2 tumour DNA was analysed further by Southern blot hybridization after cleavage with HindIII, BamHI or EcoRI endonucleases, which do not cleave the c-myc gene (Gazin et al., 1984). Hybridization with an HPV-16 DNA probe disclosed a highly labelled band with a size of about 50 kb and a minor band of 6 kb for HindIII, a non-cutting enzyme for HPV-16. After cleavage with BamHI (which cuts HPV-16 DNA once) and EcoRI (which cuts HPV-16 DNA twice), at least five and eight fragments, respectively, were detected, indicating a complex integration pattern (Fig. 6, lanes 1, 5 and 9). None of these bands was revealed with the c-myc exon 1, 2 or 3 probes, as illustrated for the c-myc exon 1 and 3 probes (Fig. 6).

A DNA library was constructed from BamHI-restricted IC2 DNA in a bacteriophage λ vector and the recombinant plaques were screened successively with the HPV-16 probe and the c-myc exon 3 probe, which detects all rearranged c-myc DNA fragments (Fig. 6). Of the 10^5 plaques analysed, none hybridized with both the HPV-16 and c-myc probes. Taken together, these data do not support the suggestion that integration of HPV-16 sequences occurred within or in the close vicinity of the c-myc gene in the IC2 tumour.

Insertion of cellular sequences within c-myc intron 2 in the IC2 tumour

Southern blot hybridization of IC2 DNA cleaved with the three non-cutting enzymes for the c-myc gene using c-myc exon 1, 2 or 3 probes showed extra bands with sizes of 15 (HindIII), 14 (BamHI), 7 or 6.5 kb (EcoRI) compared with the fragments detected in human placenta DNA (Fig. 6, lanes 2–4, 6–8 and 10–12). Furthermore, an additional highly labelled band of 7 (HindIII), 8 (BamHI) or 3 kb (EcoRI) was detected with the exon 3 probe (Fig. 6, lanes 4, 8 and 12). The DNA library constructed from BamHI-restricted IC2 DNA allowed further characterization of the rearranged and amplified sequences.
Recombinant λ phages isolated from six plaques hybridizing with the c-myc exon 3 probe were all found to contain an 8 kb BamHI fragment. None contained the abnormal 14 kb fragment detected by all c-myc probes. A 2.9 kb BamHI–EcoRI fragment containing the c-myc sequences was subcloned and sequenced. This fragment was found to comprise a 399 bp non-coding sequence inserted upstream of c-myc sequences (2612 bp) encompassing most of intron 2, exon 3 and the downstream non-coding sequences (Fig. 7). The 399 bp segment showed no significant similarity to the c-myc gene or to any of the sequences available in the EMBL database. The junction between the unknown sequences and the c-myc gene was mapped to nucleotide 5471 in intron 2. Only one nucleotide change was observed (C\textsuperscript{7775}→T), located downstream of c-myc exon 3, compared with the published sequence (Gazin et al., 1984).

Discussion

Integration sites for HPV sequences in primary genital carcinomas or derived cell lines have been mapped to chromosomal bands 1q21–q23 (Koopman et al., 1999), 3p14 (Wilke et al., 1996), 3p21 (Cannizzaro et al., 1988), 3q26–q29 (Koopman et al., 1999), 8q21–q23 (Cannizzaro et al., 1988; Gallego et al., 1994), 12q14–q15 (Sastre-Garau et al., 1995), 13q21 (Mincheva et al., 1987) and 18q21 (Reuter et al., 1998) or near translocation breakpoints involving chromosomes 1q and 22q, 3p and 13q and 3p and 14 (Koopman et al., 1999), in addition to chromosome bands 8q24 and 2p24 containing myc genes (Couturier et al., 1991; Dürst et al., 1987; Hori et al., 1991; Lazo et al., 1989; Popescu et al., 1990). The impact of the integration event on the structure and expression of cellular genes possibly involved in tumour progression is still poorly documented. Integration of HPV-16 in the CC7T-α cell line led to the disruption of the cellular Jun-B gene and a hybrid transcript was found to be expressed from the c-Jun promoter and to contain 5′ c-Jun coding sequences and sequences from the L1 ORF (Choo et al., 1995). Integration of HPV-68 sequences took place upstream of the first coding exon of the APM-1 tumour suppressor gene in ME180 cells and a polycistronic fusion transcript was shown to be expressed from the early HPV-68 promoter and to contain HPV-68 E6 and E7 and APM-1 coding sequences (Reuter et al., 1998).

In IC4 tumour cells, subgenomic HPV-45 DNA sequences were found to be integrated within the 3′ untranslated part of N-myc exon 3, 15 nucleotides upstream of the bona fide polyadenylation signal, with a 10- to 20-fold amplification of both N-myc and viral sequences. The HPV-45 genome was interrupted in the 3′ part of the L2 ORF and in the 5′ part of the E1 ORF, a pattern similar to that described recently for HPV-45 in the cervical carcinoma-derived MS751 cell line (Geisbill et al., 1997). Despite the significant association of HPV-45 with invasive cervical carcinoma (Bosch et al., 1995) and its worldwide distribution (Stewart et al., 1996), data on the intratype variability of the E6 and E7 ORFs were available for two isolates only, the prototype (Myers et al., 1994) and an isolate described by J. B. Kaplan and R. D. Burk (unpublished EMBL accession no. M38198). Alignment of the deduced amino acid sequences of IC4 HPV-45 oncoproteins with those of the two isolates disclosed a total of five variable amino acid positions in the E6 protein (residues 8, 10, 30, 53 and 118) and four in the E7 protein (residues 3, 5, 6 and 82). High levels of transcripts of the E6 and E7 region were detected in IC4 cells, supporting a role for the viral oncoproteins in the carcinogenesis process. In addition, a fusion ORF was created at the 3′ virus–cell junction that encodes a putative 153 amino acid protein including the 120 amino terminal residues of the viral E1 protein.

IC4 cells express high levels of N-myc transcripts processed via cryptic polyadenylation signals located upstream of the 5′ junction, as well as a minor N-myc/HPV-45 fusion transcript that terminates within the viral URR. It seems unlikely that these high levels of N-myc transcripts result from an increased half-life, since integration did not delete the U-rich region of the 3′ untranslated part of the transcripts, a region found to be responsible for the rapid cytoplasmic turnover of c-myc transcripts (Jones & Cole, 1987). Since the N-myc protooncogene is not expressed in the normal cervical epithelium (Couturier et al., 1991), its activation could result from insertional mutagenesis, possibly enhanced by the co-amplification of N-myc and of HPV-45 sequences, or from the amplification of the N-myc gene per se. Integration of viral sequences within the N-myc gene and the subsequent cis-activation of the protooncogene have commonly been observed in T cell lymphomas induced by the Moloney murine leukaemia virus (van Lohuizen et al., 1989) and in hepatocarcinomas associated with woodchuck hepatitis virus (WHV) (Fourel et al., 1990). The epithelium-specific enhancer identified in the HPV-18 URR (nt 7579–7738) (Thierry, 1993) is highly conserved in the HPV-45 genome (nt 7578–7737) (Myers et al., 1994). Insertion of this putative enhancer sequence about 9 kb downstream of the N-myc promoter in IC4 cells could possibly lead to its cis-activation. Transient transfection assays with the chimeric IC4 N-myc/HPV-45 sequence would demonstrate such a mechanism, as already shown for N-myc2–WHV constructs (Wei et al., 1992). On the other hand, the 10- to 20-fold amplification of the N-myc gene by itself could also account for the overexpression of the N-myc gene in IC4 cells, as observed in neuroblastoma (Brodeur et al., 1984; Schwab et al., 1984), retinoblastoma (Lee et al., 1984) and small-cell carcinoma of the lung (Nau et al., 1986). Comparable large amounts of N-myc protein were detected in IC4 cells and in SKNBE neuroblastoma-derived cells, which show a 50-fold amplification of the N-myc gene (de Créouy et al., 1997). The slightly faster migration of IC4 N-myc protein may indicate that this protein corresponds to the non-phosphorylated form (Mákelä et al., 1989). The effects of phosphorylation on the functions of myc proteins (transactivation, DNA binding,
interaction with Max protein) are far from understood (Lüscher & Larsson, 1999). It is worth stressing that mutations affecting two phosphorylation sites in the transactivation domain of the c-myc protein have been shown to result in a higher transforming potential (Nesbit et al., 1999). Since myc proto-oncogenes encode transcription factors that regulate the expression of genes involved in cell proliferation and apoptosis (Nesbit et al., 1999), it is likely that the activation of N-myc played a part in tumour progression.

Amplification, rearrangement and/or overexpression of the c-myc gene were reported to be frequent in invasive cervical carcinomas (Bourhis et al., 1990; Ocacid et al., 1987; Riou et al., 1985). Whether these genetic alterations are related to the insertion of HPV sequences is still unknown. No evidence for the integration of HPV-16 sequences in or in the close vicinity of the c-myc gene was obtained for IC2 cells. A major c-myc rearrangement identified in IC2 tumour cells involved the insertion of non-coding cellular sequences within the 5′ part of intron 2 and the amplification of the insert and the downstream c-myc sequences. In the absence of abnormal expression of the c-myc gene (Couturier et al., 1991), it remains to be determined whether the integration of HPV-16 in band 8q24.1 in IC2 cells resulted in the abnormal expression of another as yet unidentified gene possibly involved in tumour progression. That c-myc amplification and/or overexpression can occur independently from virus infection in virus-associated cancers has been shown in Burkitt’s lymphoma cells associated with the Epstein–Barr virus (Magrath, 1990) and in hepatocarcinomas induced by the ground squirrel hepatitis virus (Transy et al., 1992). However, our data on IC4 cells provide evidence for a possible role of integration of HPV sequences in cervical carcinogenesis through a mechanism of insertional mutagenesis and/or gene amplification.

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