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Analysis of the Biological Role of Human Papilloma Virus (HPV)-Encoded Transcripts in Cervical Carcinoma Cells by Antisense RNA

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A. Introduction

In cervical carcinomas and cell lines derived from them, human papilloma virus type 16 or 18 DNA has been found integrated into the host cell genome [6]. The chromosomal localization differs in different cell lines, as has been shown by in situ hybridization techniques (Mincheva et al., submitted for publication). The circular viral DNA is always disrupted in the E1/E2 open reading frame (ORF) by the integration event, and parts of the late viral genes can be deleted [6]. In contrast, the noncoding region containing promoter and enhancer sequences and the ORFs E6 and E7 are preserved. Northern blot analysis revealed that in all cell lines tested so far these ORF E6 and E7 are consistently transcribed into mRNA. Sequence analysis of cDNA clones derived from the three HPV 18-positive cell lines HeLa, SW 756, and C4-I revealed that the mRNAs consist of a 5′ viral sequence and a 3′ cellular sequence encoded by the flanking host cell DNA. Only the viral sequences give rise to major ORFs which may code for three putative proteins: E6, E7, and a spliced form of E6 (E6*) [5]. Therefore, it was speculated that these proteins are required for the characteristic growth pattern of these cervical carcinoma cells.

To analyze the biological role of the putative proteins within the cells we tried to specifically inhibit the translation by antisense RNA. The E6 and E7 ORFs were cloned in inverse orientation into a eukaryotic expression vector and transferred into HPV 18-positive C4-I cervical carcinoma cells. Preliminary data suggest that expression of HPV 18 E6 and E7 antisense RNA may lead to inhibition of cell growth.

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B. Results

The antisense RNA technique proved to be an intriguing approach to study the function of certain genes. Introduction of complementary RNA by expression vectors led to specific inhibition of gene expression and phenotypic conversion to "minus mutants" in several eukaryotic cells [2]. It was shown that complementary RNA strands hybridize within the cells, thereby inhibiting a normal nucleocytoplasmic transport and formation of an intact translation initiation complex [3, 4]. The excess of antisense RNA appears to be critical in such experiments.

To determine the influence of specific inhibition of HPV 18-encoded early mRNAs in HPV 18-positive cervical carcinoma cell lines, C4-1 cells were used as the test system; these cells contain only one viral genome copy per cell and express HPV 18 transcripts at a low level compared with other cell lines [6].

Expression vectors were constructed as shown in Fig. 1. The strong cytomegalovirus (CMV) immediate early promoter and enhancer element [1] (Pawlita, personal communication), was chosen to direct transcription of HPV 18 E6 and E7 sense or antisense mRNA. The HPV sequences were derived from a cDNA clone of the HPV 18-positive

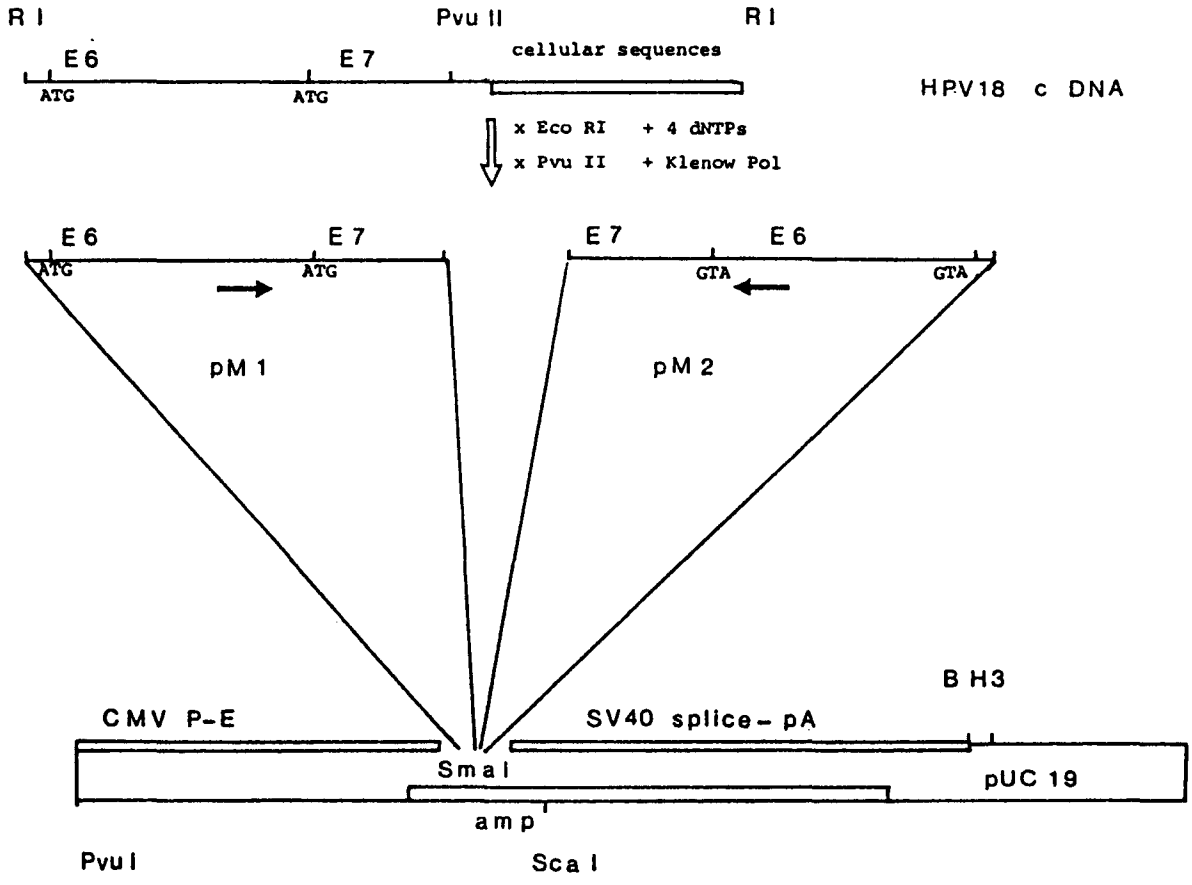

Fig. 1. Construction of expression vectors. Into the *Bam*HI site of a pUC19-derived plasmid containing the HCMV promoter (*P*) and enhancer (*E*) element (kindly provided by M. Pawlita) the *Bgl*II-*Bam*HI fragment derived from the pSV2 β-globin vector containing the SV40 early RNA-processing signals was cloned. The HPV 18 fragment derived from the cDNA clone (see text) was inserted into the *Sma*I site by blunt – end ligation. Clones with the HPV 18 E6 and E7 ORFs in both orientations were received and referred to as pM1 (sense) vector or pM2 (antisense) vector

cell line SW 756 [5]. This clone was used because it contains all HPV 18 E6 and E7 sequences in an unspliced form and covers 40 base pairs of the nontranslated 5′ sequence upstream from the E6 ATG start codon. At the nontranslated 5′ region the initiation complex is formed. Hybrid formation of the mRNA with a complementary RNA strand in this particular area was shown to be very important in other systems where antisense RNA was used to suppress gene expression [4]. To avoid interactions with other cellular transcripts, the cellular flanking sequence at the 3′ end of the E7 ORF was removed at the *Pvu*II site (see Fig. 1). The remaining HPV 18 sequences were cloned in both orientations downstream from the promoter and enhancer element. RNA processing signals were derived from the SV40 early region. Before transfection, performed by the calcium phosphate technique, the DNA was linearized at the *Sca*I site which is distant to the transcription unit. It was either used as a monomer or ligated to oligomeres before introduction into the cells. To select transfected cell clones, a dominant marker plasmid (pSV2neo) was co-transfected in a ratio of 1 : 20, and culture dishes were screened for G418-resistant colonies.

When 20 μg pM2 antisense vector DNA (cut and ligated to oligomeres) were co-transfected with 1 μg pSV2neo DNA into 2.5×10^6 C4-1 cells and incubated in DMEM medium containing 10% FCS and 800 μg/ml G418, no surviving cells were found after 4 weeks (Table 1). After transfection of unligated pM2 antisense vector DNA one G418-resistant cell clone could be raised. When the sense mRNA expressing construct pM1 was transfected into C4-1

Table 1. Numbers of G418 – resistant cell clones in the different transfection experiments. Culture dishes were screened for colonies 4 weeks after transfection

C4-I carcinoma cells	pM1	pM2
DNA cleaved with *ScaI*	15	1
DNA cleaved with *ScaI* and ligated to oligomeres	40	0
C127 mouse fibroblasts	**pM1**	**pM2**
DNA cleaved with *ScaI*	5	7
DNA cleaved with *ScaI* and ligated to oligomeres	5	11

cells following the same protocol, 40 and 15 G418-resistant colonies formed respectively. Six of each group were raised to cell lines.

In control experiments HPV 18-negative C127 mouse fibroblasts were treated by the same protocol. As shown in Table 1, the same number of G418-resistant colonies formed when these cells were transfected with either ligated or unligated sense pM1 or antisense pM2 DNA.

Southern blot analysis of the one C4-I clone transfected with the pM2 antisense vector showed that the antisense RNA transcription unit is integrated in a rearranged pattern, while in C4-I clones transfected with the pM1 sense construct the transcription unit was not found to be rearranged. The C127 fibroblasts contained unrearranged transcription units after transfection of the sense or antisense plasmid. These data may suggest that expression of antisense RNA to HPV 18 transcripts in C4-I cells led to diminished cell viability.

To test whether this effect was due to the expression of antisense RNA in these cells we exchanged the CMV promoter and enhancer element with the inducible mouse mammary tumor virus long-terminal repeat. Equal numbers of G418-resistant cell clones grew out after transfection of the inducible sense or antisense RNA-expressing vectors. We are presently analyzing the DNA and RNA of these clones, as well as possible effects of dexamethasone induction of the expression vectors on the growth characteristics of these cervical carcinoma cells.

C. Conclusions

The transfection experiments indicate that expression of antisense RNA to HPV 18 early transcripts in HPV 18-positive cervical carcinoma cells may lead to decreased growth properties of the cells. HPV 18-negative cells are not influenced by expression of sequences complementary to HPV 18 mRNA.

Because an apparently nonviable phenotype was rendered in this first set of experiments, it was not possible to correlate expression of antisense RNA to decreased growth rates of the C4-I cells directly. This could be achieved if expression of antisense RNA is directed by an inducible promoter element. It will be interesting to analyze whether gene expression of HPV 18 early ORFs E6 and E7 acts in a dose-dependent manner on the growth characteristics of cervical carcinoma cells.

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