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Specific interaction between hnRNP H and HPV16 L1 proteins: Implications for late gene auto-regulation enabling rapid viral capsid protein production

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ABSTRACT

Heterogeneous nuclear ribonucleoproteins (hnRNPs), including hnRNP H, are RNA-binding proteins that function as splicing factors and are involved in downstream gene regulation. hnRNP H, which binds to G triplet regions in RNA, has been shown to play an important role in regulating the staged expression of late proteins in viral systems. Here, we report that the specific association between hnRNP H and a late viral capsid protein, human papillomavirus (HPV) L1 protein, leads to the suppressed function of hnRNP H in the presence of the L1 protein. The direct interaction between the L1 protein and hnRNP H was demonstrated by complex formation in solution and intracellularly using a variety of biochemical and immunochemical methods, including peptide mapping, specific co-immunoprecipitation and confocal fluorescence microscopy. These results support a working hypothesis that a late viral protein HPV16 L1, which is down regulated by hnRNP H early in the viral life cycle may provide an auto-regulatory positive feedback loop that allows the rapid production of HPV capsid proteins through suppression of the function of hnRNP H at the late stage of the viral life cycle. In this positive feedback loop, the late viral gene products that were down regulated earlier themselves disable their suppressors, and this feedback mechanism could facilitate the rapid production of capsid proteins, allowing staged and efficient viral capsid assembly.

1. Introduction

hnRNP H is a member of the heterogeneous nuclear ribonucleoprotein (hnRNP) family. hnRNP H has been shown to form heterodimers during the regulation of mRNA splicing [1,2]. During this process, hnRNP H binds to intronic G triplets or G3: for example, the six "GGG" repeats in RNA as shown in Fig. 1A [3,4]. hnRNP H enhances the binding of cleavage/polyadenylation specificity factor (CPSF) to RNA polyadenylation sites [5]. This binding event leads to further enhancement of pre-mRNA cleavage and polyadenylation [6–8]. As previously reported [3,9], in mRNA, the polyadenylation site sequence procedes (i.e., is 5′ to) the GU-rich sequence, which includes several intronic G triplets. The binding of CPSF to the polyadenylation site failed to stimulate cleavage and polyadenylation [10], demonstrating that the binding of hnRNP H is required to initiate cleavage and polyadenylation [3,9]. In several viruses, hnRNP H has been shown to play critical roles in the splicing regulation of several viral mRNAs, including the splicing of simian vacuolating virus at 40 pAL [11], human papillomavirus (HPV) at pAE [3], Rous sarcoma virus pre-mRNA [12] and HIV type 1 tat-specific exon 2 and tev-specific exon 6D [13,14].

Over 100 serotypes of HPV exist, with more than 50 serotypes being human pathogens [15]. The most studied serotype, HPV16, is a high-risk serotype and the most common serotype that causes cervical cancer in women. In all HPV serotypes, there are two late viral proteins (L1 and L2) that form the viral capsid, with L1 being the predominant protein. The HPV16 L2 sequence encodes a polyadenylation element, which encompasses multiple GGG (or G3) motifs in the RNA. In addition, hnRNP H has been shown to interact with the G3-containing motifs, and it is speculated that hnRNP H regulates polyadenylation at the HPV16 early polyadenylation signal, which suppresses the expression of the late proteins during the early stage of the viral life cycle [3]. Whether this regulatory protein interacts with late gene products (once expressed) at the protein level, providing a feedback loop, remains unclear.

In this study, we assessed the possible association between the HPV L1 capsid protein and hnRNP H, the suppressor of the late gene expression. To ensure that the reporter module pPG was suitable for this purpose, the tuning of hnRNP H activity using RNAi

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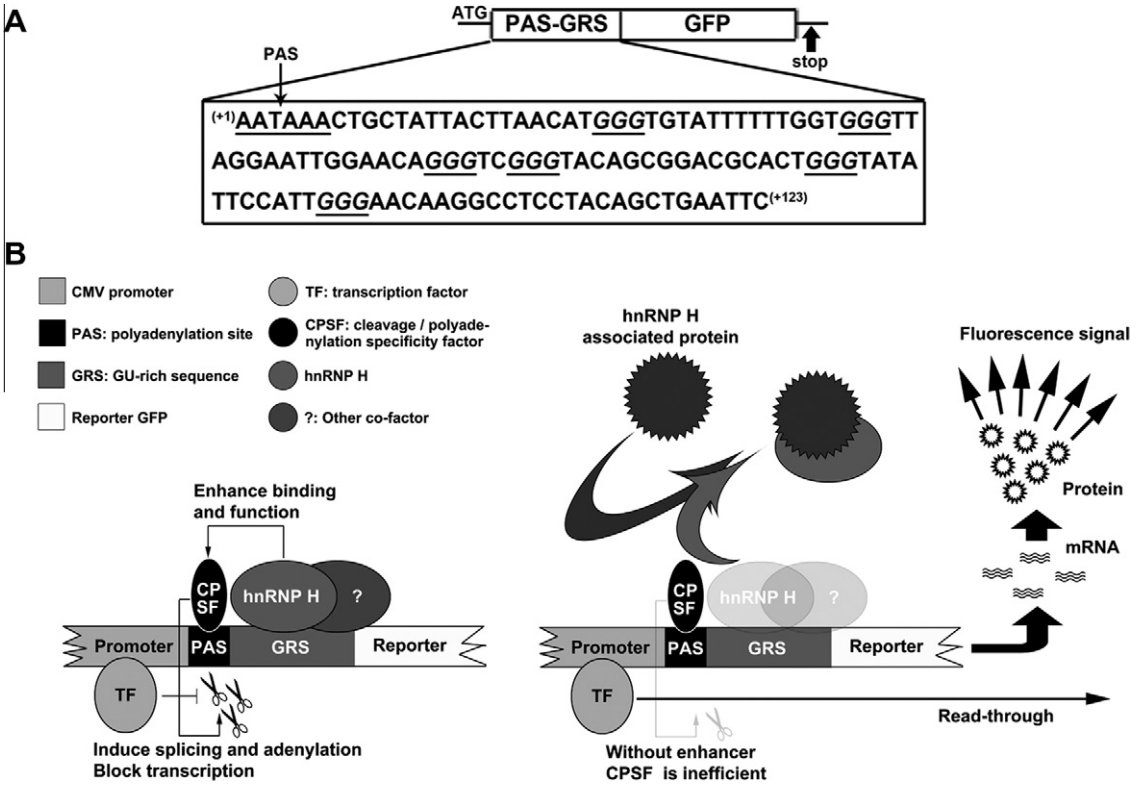

Fig. 1. Construction of the reporter plasmid pPG using GFP as a reporter (A) for functional analysis of hnRNP H activity and the envisioned mechanism of the RNA-binding proteins during gene regulation (B). The plasmid contains a strong CMV promoter, polyadenylation site (PAS), GU-rich sequence (GRS) and a green fluorescence protein (GFP) reporter, as shown in Fig. 1A. There are three differences between the pPG used in this study and the plasmid described by Oberg et al. [3]: (a) only the key sequence AAUAA was introduced instead of the full-length pAE sequence; (b) the HPV16 L1 and L2 genes were replaced by the GFP reporter gene; and (c) a synthetic GRS signal sequence was used in the absence of the L2 genes.

and other means was demonstrated with the varying level of the resultant green fluorescent protein (GFP) gene or protein. Once validated, the impact of HPV16 L1 co-expression with pPG was studied, and this co-expression demonstrated effective suppression of hnRNP H activity in the presence of the L1 protein. Moreover, the direct association between HPV16 L1 and hnRNP H was demonstrated on a molecular level by various techniques, including peptide mapping after specific pull-down experiments, co-immunoprecipitation and intracellular co-localization.

2. Materials and methods

2.1. Cell lines, proteins, and antibodies

HeLa cells (ATCC) were cultured with RPMI-1640 (GIBCO) containing 10% fetal calf serum at 37 °C with 5% CO_2. Recombinant HPV16 L1 self assembles into virus-like particles upon over-expression in various host cells, including *E. coli* [16], bacuolovirus [17] and yeast [18,19]. *E. coli*-expressed HPV16 L1 [20] was used for specific pull-down analysis. The HPV16 L1-specific mouse monoclonal antibodies (mAbs), denoted PB9 and PB11, were produced in-house using hybridoma technology. The purified IgG form of PB9 and PB11 was used. The rabbit anti-His and anti-GFP polyclonal antibodies (pAbs) and mouse anti-β-tubulin mAb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). FITC-GAM-IgG and TRITC-GAR-IgG were purchased from Sigma–Aldrich Corporation (St. Louis, MO). IR Dye 800CW-conjugated goat (polyclonal) anti-rabbit and anti-mouse IgG were purchased from Invitrogen (Carlsbad, CA).

2.2. Plasmid construction

2.2.1. Reporter pPG plasmid and plasmid for hnRNP H expression

As shown in Fig. 1A, the polyadenylation site sequence (PAS) and GU-rich sequence (GRS) with six G-triplets, serving a similar function with early polyadenylation site (pAE) in HPV, were constructed upstream or at the 5′ to the GFP reporter gene. In the HPV genome, the GU-rich sequence was found in the part of the 5′ coding region for HPV16-L2 [3]. The PAS-GRS sequence was inserted into pcDNA3.1 at *BamHI* and *EcoRI* sites. The GFP sequence was amplified by PCR and inserted into pcDNA3.1-pAE-G at *EcoRI* and *XhoI* sites to generate pPG. The pHis-H-hnRNP H sequence was amplified by PCR using Pvl1392 H as a template with the primers H-F and H-R. All of the plasmids and primers used in the plasmid construction are listed in Supplementary Tables 1S and 2S.

2.2.2. Inhibitory plasmids for the investigation of hnRNP H activity

The oligo GGGCACAGGTATATTGAAA for RNAi of hnRNP H was designed using an algorithm (http://www.dharmacon.com/sidesign) and inserted into a pSUPER vector purchased from OligoEngine (Seattle, WA) to construct pSUPER-Hi by following established protocols. In brief, two complementary oligos, Hif and Hir, were designed and synthesized. The oligos were mixed and denatured at 90 °C for 10 min and then annealed at room temperature for 30 min to generate sticky ends at both the 3′ and 5′ ends. The annealed product was subsequently inserted into pSUPER at the *BglII* and *HindIII* sites to generate pSUPER Hi. The plasmid p16L1 h, containing the HPV16 L1 expression cassette [20], was transfected into mammalian cells to co-express HPV L1.