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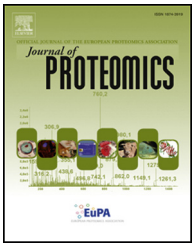

Biochemical and proteomic analysis of spliceosome factors interacting with intron-1 of human papillomavirus type-16☆

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ABSTRACT

The human papillomavirus type 16 (HPV-16) E6/E7 spliced transcripts are heterogeneously expressed in cervical carcinoma. The heterogeneity of the E6/E7 splicing profile might be in part due to the intrinsic variation of splicing factors in tumor cells. However, the splicing factors that bind the E6/E7 intron 1 (In-1) have not been defined. Therefore, we aimed to identify these factors; we used HeLa nuclear extracts (NE) for in vitro spliceosome assembly. The proteins were allowed to bind to an RNA/DNA hybrid formed by the In-1 transcript and a 5′-biotinylated DNA oligonucleotide complementary to the upstream exon sequence, which prevented interference in protein binding to the intron. The hybrid probes bound with the nuclear proteins were coupled to streptavidin magnetic beads for chromatography affinity purification. Proteins were eluted and identified by mass spectrometry (MS). Approximately 170 proteins were identified by MS, 80% of which were RNA binding proteins, including canonical spliceosome core components, helicases and regulatory splicing factors. The canonical factors were identified as components of the spliceosomal

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B-complex. Although 35–40 of the identified factors were cognate splicing factors or helicases, they have not been previously detected in spliceosome complexes that were assembled using *in vivo* or *in vitro* models.

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1. Introduction

Infection with high-risk human papillomavirus (hrHPV) causes hyper-proliferative lesions in mucosa, which in turn might be transformed into malignant neoplasias [1]. The hrHPV E6/E7 oncoproteins play a pivotal role in the malignant transformation of cervical epithelium and the underlying mechanism is well documented [2,3]. The E6/E7 oncoproteins are encoded by neighboring genes, which are expressed as polycistronic primary transcripts (pre-mRNAs) [4,5]. Interestingly, E6/E7$^{\text{pre-mRNAs}}$ are spliced in hrHPVs, but this does not occur in the low-risk HPVs [6]. HPV type 16 (HPV-16) is the most prevalent infection in cervical cancers. It is found in 60% of the cases, suggesting its major role in the development of this cancer [7]. Intriguingly, their pre-mRNAs are alternatively spliced, whereas E6/E7$^{\text{pre-mRNAs}}$ from other hrHPVs are processed by *cis* splicing [8]. Four isoforms of the HPV-16 E6 mRNA are produced by alternative splicing [9,10]. These reports suggest that the E6 splicing products might play an important role in the development of cervical cancer; however, this hypothesis is yet to be confirmed.

HPV introns are recognized and processed by the host cell splicing machinery, namely the spliceosome, which is a multi-protein complex formed by 5 small nuclear ribonucleoprotein particles (snRNPs), namely U1 to U6 snRNPs, each of which are formed with a set of specific proteins and their corresponding snRNAs [11]. Together with the snRNPs, a number of accessory factors are assembled in the pre-mRNA to constitute a catalytically active spliceosome [reviewed in 12]. The spliceosome is assembled stepwise by an ordered multi-step process and at least four intermediary particles might be fractionated by biochemical procedures, namely the E-, A-, B-, and C-complexes. The E-complex is constituted by the pre-mRNA plus the U1 snRNP; it is involved in the recognition of the splicing donor sequence. In turn, U2 snRNP and its associated factors (U2AF$^{65/35}$) bind to the branch point (BP) and the acceptor sequence, to form the A-complex. After this, the tripartite particle U4/U6°U5 snRNP is bound to form the B-complex [13]. Subsequently, ATP is necessary to produce a B active-complex (B*), which in turn gives rise to the catalytically active spliceosome, the C-complex [14]. Interestingly, evidence in yeast suggests that it may also be pre-assembled [15,16]. Regardless of the assembly mechanism, at least 300 proteins have been observed in spliceosomes [17]. Among them, heteronuclear ribonucleoproteins (hnRNP), serine-arginine rich (SR) proteins, protein kinases, cyclophillins, GTPases and RNA helicases [17,18].

The HPV-16 E6/E7 intron 1 (In-1), contains one donor splice site and three suboptimal acceptor sites. In-1 is distinct because it is embedded in the coding sequences from both, the E6 and E7 oncogenes. Thus, the coding sequences are recognized and spliced. Noteworthy, the alternatively spliced transcripts are heterogeneously expressed in cervical carcinoma cells [10,19,20]. The heterogeneous profile seems to be promoted by two conditions: intrinsic variation in the level of some splicing factors in the tumor cells [21] or a differential binding of splicing factors to polymorphic variants of the HPV-16 [22]. Unfortunately, both the mechanisms and splicing factor that facilitate this heterogeneous expression are largely unknown. Proteomic and mass spectrometry technology might be an excellent tool to identify the nuclear factor which binds the HPV-16 In-1. In fact, this technology has been recently used to analyze human Thin Prep cervical smears from normal or cancer tissues, identifying inclusive protein from different HPV types [23].

Given this background, we were prompted to investigate the identity of the nuclear factors that bind to In-1. First, we initiated the biochemical characterization of the E6/E7$^{\text{pre-mRNA}}$–protein-complexes formed during *in vitro* splicing conditions, by using HeLa nuclear extract (NE). Subsequently, the complexes were characterized by using ultraviolet (UV) cross-linking assays under different experimental conditions. Mutation/deletion analysis of the splicing donor and acceptors suggested a differential recognition of each of the acceptor sites. Moreover, these data suggested binding of multiple protein factors – including poly-U binding proteins – which seem to be important to stabilize the spliceosomal complexes. To simplify the system, we initiated the purification of factors that were bound to the shorter In-1 (sIn 1), that is, the intron sequence encompassing only the donor and the closest acceptor sequence; because, this alternative intron is used with the higher frequency in cervical carcinoma cells. A synthetic RNA probe was used to bind and to purify the nuclear factors; the probe was annealed to a 27-nt biotinylated-DNA oligonucleotide (complementary to the 5′ end of In-1) to generate a DNA/RNA hybrid. The hybrid probes bound with the proteins were coupled to streptavidin magnetic beads for purification. The proteins were eluted and subsequently identified by liquid chromatography tandem mass spectrometry (LC/MS/MS). One hundred and thirty five of these proteins corresponded to snRNP and non-snRNP splicing factors. Approximately 95 of these factors were identified as core spliceosome components. Interestingly, 35–40 of the identified factors that were cognate splicing factors or helicases were not previously reported as spliceosome components. Moreover, some of these factors are involved in enhancing weak splicing donor and acceptor recognition in mammalian mRNAs. This article discusses the significant influence of these findings in the splicing of HPV-16 In-1.

2. Materials and methods

2.1. Reagents and primers

HeLa NE containing proteins that are active in splicing were purchased from Promega Co (Madison, WI, USA). All molecular biology reagents and primers were purchased from Invitrogen

(Carlsbad CA USA). We designed biotinylated DNA oligonucleotides which were complementary to the 5′ region of the E6/E7 RNA probes (Table 1). The magnetic beads used in this study were mMACS Streptavidin Microbeads (Miltenyi Biotec; Gmbh).

2.2. Constructs and preparation of pre-mRNA substrates

The pHPV-16-E6/E7R plasmid (R = reference) harboring the E6/E7 open reading frames (ORFs) was used as the DNA template to generate all of the constructs used in this study [22]. Amplicons were synthesized by PCR, cloned into a T-protruding vector (pBS SK+), and sequenced. Constructs C1, C2, C3, and C4 were prepared by using the primer HPV-16R combined with primers CIHPV-16, EIHPV-16, DIHPV-16, and BIHPV-16, respectively. The first three constructs contained the donor and 3, 2, or 1 of the acceptor sites, while the fourth contained only donor and BP sequences, but not acceptor sites; they produced synthetic RNA fragments of 741, 499, 391, and 310 nt, respectively. Constructs C5 and C6 were prepared by using the following primer pairs: FHPV-16/DIHPV-16 or GHPV-16/CIHPV-16, which contained sequences surrounding each of the splicing acceptors, namely A-SA or C-SA, respectively. They produced synthetic RNA fragments of 96 or 160 nt, respectively. Constructs C2m and C7m were amplified from wild type plasmid (pHPV-16-E6/E7R). C2m contained the shorter E6/E7 splicing cassette (nt 73 to 429) and was prepared by using the primer pair HPV-16R/HPV-16 3AR and it produces a RNA fragment of 357 nt; while C3m contains the intron-1 sequence without the donor site; it was prepared using the primer pair FE6MUT226 Eco/HPV-16 3AR, it produces a 211 nt RNA fragment. For C7m the HPV-16R/HPV16M5H primer pair was used and it produces a 147 nt DNA fragment. Constructs C4m and C5m were amplified from the previously reported mutant plasmid pE6/E7SD^{M} [21], using the following primer pairs: SA-A Ext Fw/HPV-16 3AMR and FE6MUT226 Eco/HPV-16 3AMR, respectively. Construct C6m was produced by joining the DNA fragment of construct C2m, with an amplicon that contains the mutated acceptor C; previously, a *Not I* restriction site was introduced into the B-acceptor site by using the primer pair HPV-16R/HPV-16A3SSMR. Subsequently, the amplicon was ligated to a DNA fragment amplified with the following primer pair: HPV-16A3SSM/HPV-16B3SSM. The amplicon produces a 450 nt RNA fragment. For construct C3m the donor site was substituted by the *Hind III* restriction site; for C4m and C6m, A- and B-acceptor sites were substituted by the *Not I* restriction site, while for C5m donor and acceptor sites were substituted by the *Hind III* and *Not I* restriction sites, respectively. See Table 1 for primer sequences and Fig. 2 for diagrammatic representations of the constructs. In addition, a T7 promoter sequence was added to some of the forward primers to generate RNA products directly from the amplicons. Synthetic RNAs were labeled with 40 μCi of [α-32P] UTP during *in vitro* transcription, by using T7 RNA polymerase (Promega transcription kit). Constructs were sequenced by using the Big Dye Terminator Ready Reaction Kit and analyzed in the ABI PRISM 310 Genetic analyzer System (Perkin-Elmer; Branchburg, NJ, USA). The U2AF65 ORF was amplified by reverse transcription (RT)-PCR using total HeLa cDNA with primers that were designed from the GenBank database sequences (ID: X60648). The PCR-synthesized DNA fragment was cloned in a T-protruding vector, sequenced and subcloned at the *Bam*HI/*Xho*I restriction sites of the plasmid Proex B (Gibco-BRL). The PTB ORF was cloned in the *Hind*III site of this plasmid [24]. All His-tagged recombinant proteins were produced in the *Escherichia coli* BL21 strain and purified by Ni-NTA chromatography (QIAGEN Inc.) following the procedure recommended by the manufacturer.

2.3. RNA–protein binding and UV cross-linking assays

RNA/protein complexes were generated at 30 °C by using splicing buffer (RNA splicing system; Promega). ATP and magnesium ions were excluded to avoid splicing reactions and to make rich the assay in assembled spliceosomes. Briefly, 5 μg of HeLa NE and 5 ng of the labeled RNA probe were incubated for 20 min at 4 °C in 10 μL of splicing buffer (5 mM HEPES, pH 7.9, 0.6% PVA, 20 mM creatine phosphate, 10 % glycerol, and 0.4 mM ATP), supplemented with 2.5 mg/mL yeast tRNA. Samples were irradiated for 10 min using a 254-nm UV lamp

Table 1 – Oligonucleotide primers used for preparation of mutation and deletion constructs. Restriction sites or T7 promoter sequences introduced in PCR primers are indicated in italic font.

Oligonucleotide name	Sequence (5′-3′)	nt position
1) E6 BEGIN	CAGACATTTTATGCACCAAAAGAGAACTGCAA	83–104
2) C1HPV-16	CATTAACAGGT CTTCCAAAGTACG	813–790
3) EIHPV-16	CTCCATGCATGATTACAGCTG	571–551
4) DIHPV-16	GTCCAGATGTCTTTGCTTTTC	463–443
5) B1HPV-16	GTTGTATTGCTGTTCTAATGTTGT	382–359
6) FHPV-16	taatacgactcactatagGAACAGCAATACAACAAACCG	368–388
7) HPV-16B3SST7	taatacgactcactatagCTGTCAAAAGCCACTGTGTC	421–437
8) GHPV-16	taatacgactcactatagCTCAGAGGAAGGAGGATGAAATA	654–675
9) HPV-163AR	GGCTTTTGACAGTTAATACACCT	429–407
10) FE6mut226Eco	gaattcGTATATGACTTTGCTTTTCGG	227–247
11) HPV-16A3SSMR	ttt*gcggccgc*AATTAACAAATCACACAACGGTG	417–384
12) HPV-16M5H	*aagctt*CGTCGCAGTAACTGTTGC	222–205
13) SA-A Ext Fw	taatacgactcactataggAACAGTTACTGCGACGTGAGGTATATG	207–233
14) HPV-16R	taatacgactcactatagGACTCACTATAGCAGACATTTTATGCACAAAGA	73–95
15) HPV-16A3SSM	tttgcggccgcAACTGTCAAAAGCCACTGTGTC	407–437
17) HPV-16B3SSMR	tttgcggccgcAACAAGACATACATCGGACCGGTC	449–320
18) HPV-16B3SSM	tttgcggccgcAGAACACGTAGAGAAAACCC	522–552
19) SA-A Rv	CAGGCACAGTGGCTTTTG	440–422

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located 5 cm above the sample [22]. Samples were digested with 2 mg/mL RNAase A. Electrophoresis loading buffer and heparin (200 μg) were added to the reaction mixture, and then boiled for 10 min, and subsequently, the proteins were fractionated by 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), by using 15 cm- or 25 cm-long gels. Gels were vacuum-dried and exposed to X-ray film for 2 to 8 days. The results presented are representative of at least 4 independent experiments.

2.4. Isolation and identification of RNA-bound proteins by RNA-Affinity chromatography

Briefly, 400 pmol of the RNA probe (234 nt) was hybridized to 500 pmol of a small 3′ end biotinylated DNA-oligonucleotide (27 nt) by using 25 μL of 10 mM HEPES, 50 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM DTT supplemented with 25 U RNAsin plus (Promega), and 0.15 μg/mL yeast tRNA. The mixture was incubated at 95 ° C for 2 min, and then chilled in ice, and annealing was allowed for 1 h at 37 °C. The DNA oligonucleotide tag was complementary to the 5′ end of the intron RNA (nt 207–233). The In-1 RNA probe (nt 207–440) contained 19 nt upstream of the donor site and 21 nt downstream of the A-acceptor site.

Apart from this, a mixture of 500 μg HeLa NE plus 100 μL of magnetic beads were mixed in a batch with 500 μL of the HEPES buffer, and was incubated for 1 h at 4 °C for blocking non-specific sites and consuming residual ATP levels in the NE. Subsequently, the annealed RNA/DNA probe was poured into the NE and magnetic beads mixture and incubated for another 1 h at 37 °C. To capture the RNA/protein complexes, the mixture was passed through a micro-column (Miltenyi Biotec) and subjected to a magnetic field. The mixture was washed four times by using 200 μL of the same HEPES buffer supplemented with 0.5% Triton X-100. Finally, the proteins were eluted twice by using 100 μL of the HEPES buffer supplemented with 1 M NaCl. Eluted proteins were resolved by 12% SDS-PAGE. Gels were stained with the Silver staining kit (SilverQuest™ Invitrogen) for electrophoresis or with Coomassie Brilliant Blue R-250 for mass spectrometry (MS). For MS analysis, the proteins were concentrated by 12% SDS-PAGE; briefly, the proteins were allowed to run on the gel for approximately 1 cm; proteins were stained and cut in two slices of 4 mm each (from 35 to 60 kDa and 60 to 200 kDa, respectively); then the slices were sent for LC/MS/MS analysis. Duplicate experiments were performed for identification by MS. To verify the identity of proteins detected by only one of the previous experiments, new duplicate experiments for the protein bands in the 40–70 kDa range were cut and sent for LC/MS/MS analysis. Proteins were identified by electrospray ionization (ESI)-LC/MS/MS analysis by using a Micromass Q-TOF spectrometer equipped with an LC Packings Nanoflow, at the Columbia Protein Core Facility (Columbia University College of Physicians & Surgeons). Tryptic peptides were extracted from acrylamide gel slices and injected into a reverse-phase LC column for stratification. Data files were processed using the MassLynx, ProteinLynx software, and PkI data were submitted to www.matrixscience.com for analysis using a Mascot algorithm and the ProteoIQ software (NuSep, Bogart GA, USA).

3. Results

3.1. Biochemical characterization of protein complexes

HPV-16 E6/E7 intron has 3 different lengths, and therefore might be processed in 3 alternative acceptor sites (A-, B- and C-acceptors), producing three E6 short transcripts plus the complete full length E6 messenger. A schematic representation of the complete E6/E7$^{pre\text{-}mRNA}$ cassette is shown (Fig. 1A). For the biochemical characterization of the nuclear factors assembled in this pre-mRNA, we used synthetic E6/E7 transcript and HeLa NE for UV cross-linking assays. The experiments were performed using splicing buffer, but in the absence of ATP. The results indicated the presence of 12 bands (Fig. 1). Control experiments are presented as supplementary material (Fig S1A to C). Since cross-linking assay detect only proteins that closely interact with the RNA, these bands probably do not correspond to the isolated proteins, while the thickness of the bands suggested the presence of proteins co-migrating in each one. These RNA–protein complexes were not altered by using different proportions of RNA probe or HeLa NE (Fig. 1A), as indicated by a representative assay with three different ratios of RNA probe and HeLa NE. A ratio of 0.4 (2 nmol of RNA probe/5 μg of NE) was used for all the assays. The E6/E7$^{pre\text{-}mRNA}$ cassette has a 42% pyrimidine (Py) content, but only a 29% G + C content, which suggests a relatively high uridine (U) proportion in this intron. Analysis of the sequence revealed that its U content was 28.1% in the full-length In-1, whereas the short In-1 contained 37.3% U (Fig. 1B). Hence, we assumed that many of the factors assembled in this intron might be poly U-binding proteins, and therefore performed the cross-linking experiments in the presence of increasing amounts of a poly-U competitor RNA (16–18 nt). The results revealed that many of the complexes were not produced in the presence of high amounts of the poly-U RNA (Fig. 1C). To further characterize the complexes, we supplemented the HeLa NE with increasing amounts of a poly-U binding protein, the recombinant U2AF65 (rU2AF^{65}) protein, in order to saturate the poly-U rich sequences and to evaluate whether the poly U-rich sequences were important for the protein binding. The results indicated that the formation of the RNA–protein complexes was impaired when the intron was saturated with high levels of rU2AF^{65} (Fig. 1D). On the other hand, as a specificity control, we used a DNA-oligonucleotide complementary to the U1 snRNA, which inhibited the binding of the U1snRNP. The results indicated that this oligonucleotide impaired only the binding of a 70 kDa protein, which had the expected molecular weight for U170k, the factor that binds the U1 snRNA (Fig. 1E). These results also suggested that protein binding impairment in the 5′ end of the intron does not have an appreciable effect in the formation of most of these complexes. This fact should be useful for designing a DNA oligonucleotide tag that could facilitate the purification of these RNA–protein complexes. Hence, we continued with the characterization of these complexes in order to obtain useful data for the subsequent purification of proteins.

The stability of the complexes was analyzed by increasing the concentration of ATP, $MgCl_2$, or KCl. First, assays were performed by using increasing concentrations of ATP. The results indicated that complexes were more stable at very low

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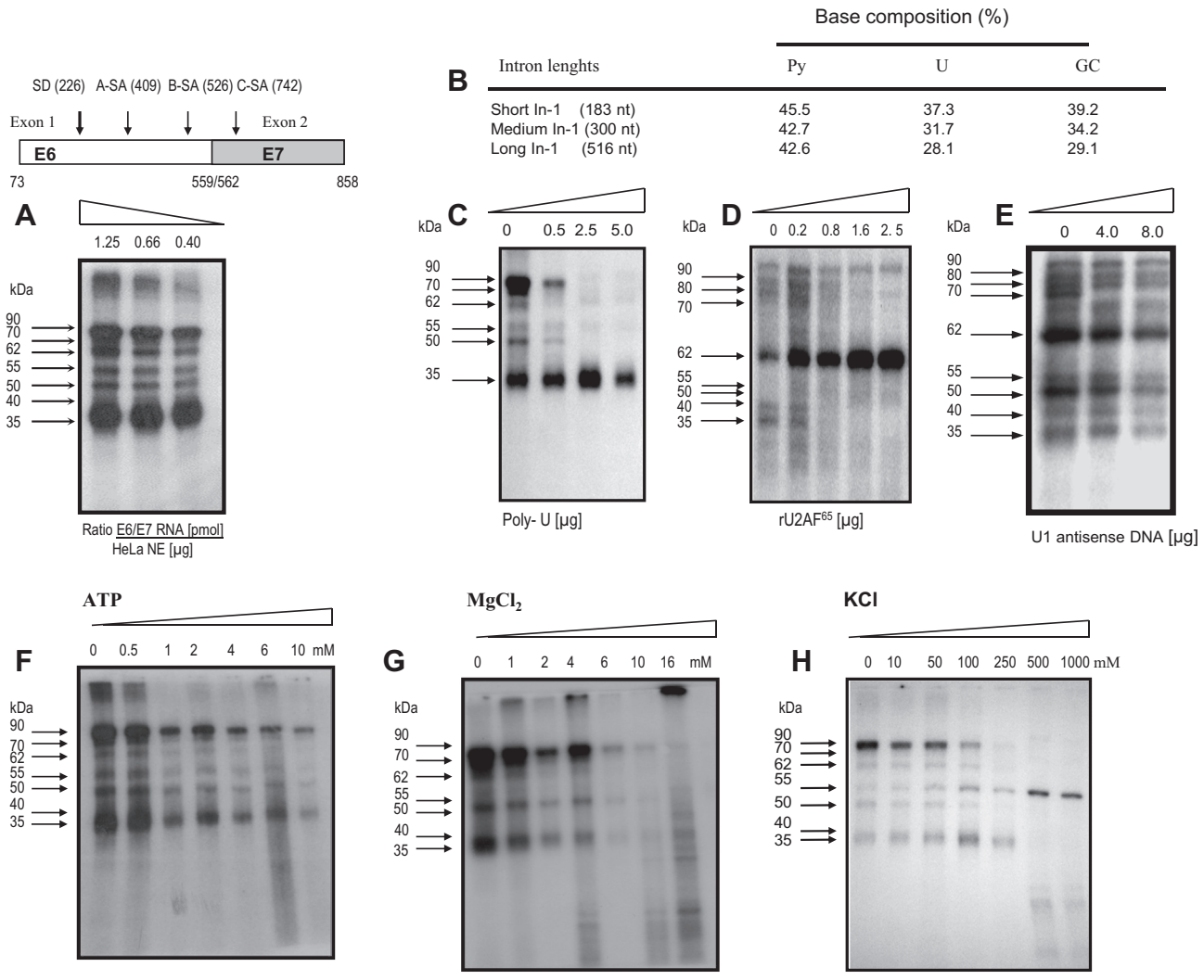

Fig. 1 – Binding pattern of HeLa nuclear proteins cross-linked to the HPV-16 E6/E7$^{pre\text{-}mRNA}$. Profile of nuclear proteins UV cross-linked to the HPV-16 E6/E7$^{pre\text{-}mRNA}$. Different proportions of HeLa NE were cross-linked to 5 ng of E6/E7 RNA containing the complete E6/E7 intron, whose structure is schematically represented (A). The E6/E7 intron (In-1) contains different proportions of uridine (U) and despite being the shortest intron, it has the highest U content (B). Addition of increasing amounts of poly-U impaired the formation of the RNA–protein cross-linking complexes (C). Addition of increasing amounts of rU2AF65 impaired the formation of the complexes (D). Addition of a DNA oligonucleotide, which competes with the binding of U1snRNP, impairs the binding of a single 70 kDa protein (E). Addition of increasing amounts of ATP (F), $MgCl_2$ (G), or KCl (H) showed that the complexes are more stable in the absence of either ATP or $MgCl_2$. Cross-linking complexes are more stable in low ionic strength (H).

concentration or in the absence of ATP (Fig. 1F). Similar results were observed in the presence of increasing amounts of $MgCl_2$; addition of more than 4 mM of Mg ions impaired the detection of the complexes (Fig. 1G). The results also indicated that the formation of the complexes was impaired at concentrations higher than 50 mM of KCl, and only a few proteins remained bound at concentrations as high as 1 M KCl (Fig. 1H). Taken together, the results suggest that the RNA–protein complexes detected by the cross-linking assays are stable in the absence of ATP and magnesium ions when they were formed at low levels of ionic strength. In addition, these complexes might be rich in poly-binding proteins.

3.2. RNA–protein complexes assembled in the shortest E6/E7 intron sequence were representative of those assembled to the complete pre-mRNA

The short intron-1 (sIn-1) is processed mainly in tumor samples and cell lines; it is used to up 8-fold more, as compared to the middle and long introns. On the other hand, the above data indicated that many poly-U binding proteins were bound to the intron in addition to the U1 snRNP. Moreover, sIn-1 was relatively richer in poly-U content than In-1. Therefore, we hypothesized that the detected RNA–protein complexes might represent mostly the proteins bound to the short E6/E7 intron.

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We performed deletion experiments in order to confirm this hypothesis. Initial experiments showed that elimination of only the donor sequence had poor effect in changing the profile of the splicing factors that bind the In-1 probe (Fig. 2A); these experiments also revealed that the deletion of the donor and BP sequences hardly changed the profile of the factors that bind the In-1 probe. The data also suggested that most of the detected proteins recognized the acceptor sequences. Moreover, progressive deletion of the two distal splicing acceptors produced minimal changes in the profile of these complexes, and most of the binding profile was conserved, even after deleting 300 nts downstream of the BP, which include the whole PyAG recognition sequences (Fig. 2B). Furthermore, mutation of the acceptor and donor sequences impaired the formation of most of the complexes, a representative experiment showing the resolution of the complexes in a 25 cm long SDS-PAGE gel, is shown (Fig. 2C). Taken together, the data confirmed that most of the characterized RNA–protein complexes were proteins that are potential spliceosomal components assembled from donor to the first acceptor sequence, and that the acceptor sequences might recognize different sets of proteins.

3.3. *snRNPs and non-snRNPs were bound to the shortest intron of E6/E7$^{pre\text{-}mRNA}$*

For further characterization, we resolved these complexes on 1% agarose gels to detect the spliceosomal complexes produced during the binding experiments in the presence and absence of UV-light. The results revealed two major bands, which are strong and corresponding to the E- and A-complexes, but a weak band (B-complex) was also detected. The results also showed that UV-light and RNAase treatments may be avoided to prevent any detrimental effects in the formation of the spliceosomal complexes; the complexes also seemed to be enriched in the absence of UV light plus RNAse A (Fig. 3A). Next, we proceeded to the purification of the proteins bound to the sIn-1. For this assay, 500 μg NE plus the streptavidin-magnetic beads were mixed with 400 pmol of the hybrid probe in 500 μL of binding buffer, to allow binding of the nuclear factors with the sIn-1 RNA; subsequently, the mix was extensively washed and the proteins were eluted, as described in the Materials and methods section. A schematic representation of the purification procedure is shown (Fig. 3B). The electrophoresis profile of the

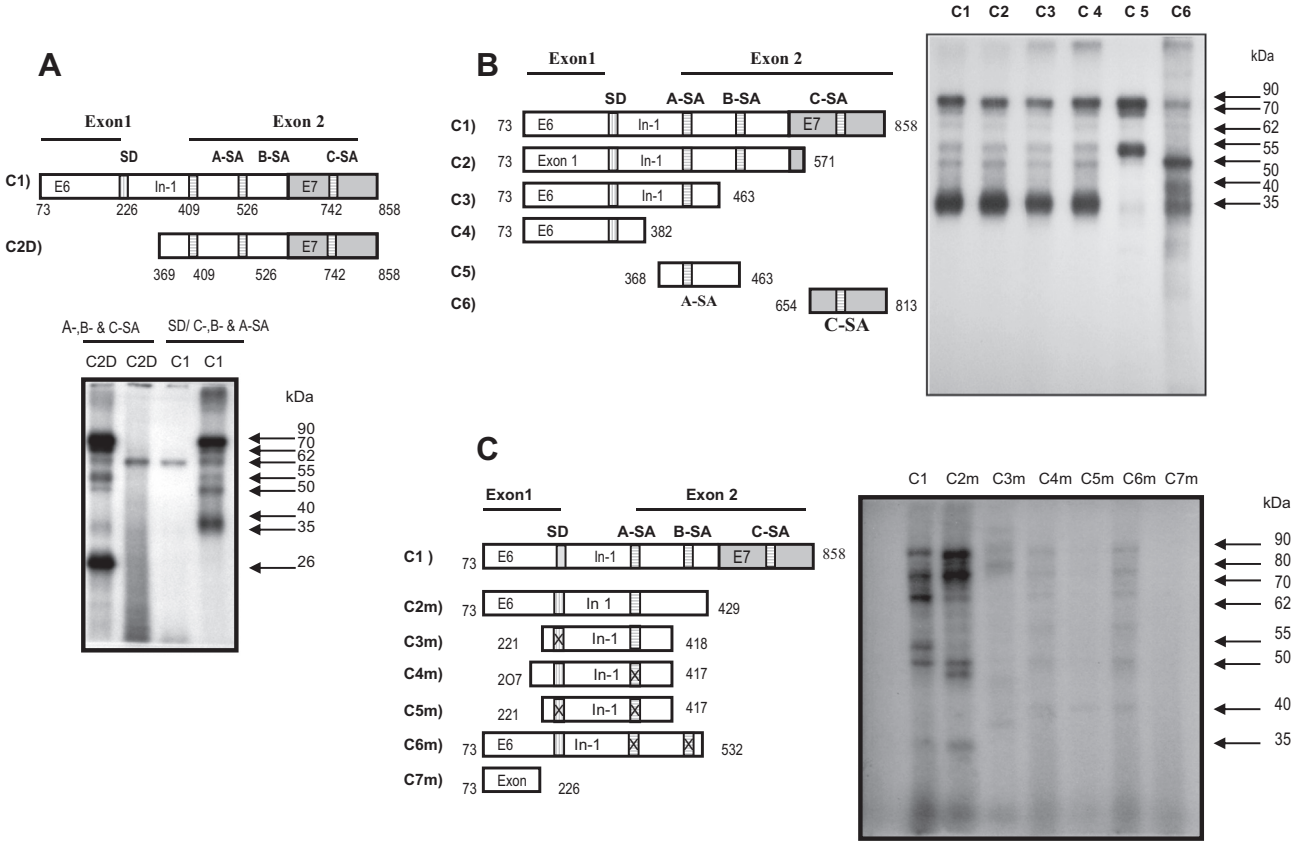

Fig. 2 – Mapping protein binding to the E6/E7$^{pre\text{-}mRNA}$. Mutated or deleted RNA probes were used to identify proteins cross-linked at splicing sites. (A). HeLa NE UV cross-linking complexes were assembled into RNA probes containing the complete intron or only branch point and the splicing acceptors are shown (lanes 1 and 4, respectively); similarly, the two RNA probes were cross-linked to 5 μ rPTB (lanes 2 and 3, respectively). Protein binding profiles of the complexes obtained by using 3′ progressively deleted RNA probes in (B) or by mutation in (C) of donor and acceptor splicing sites mutated or deleted RNA probes are schematized. Panels A and B represent proteins run using 15-cm-long SDS-PAGE gel, while panel C represent proteins run using a longer (25 cm) gel.

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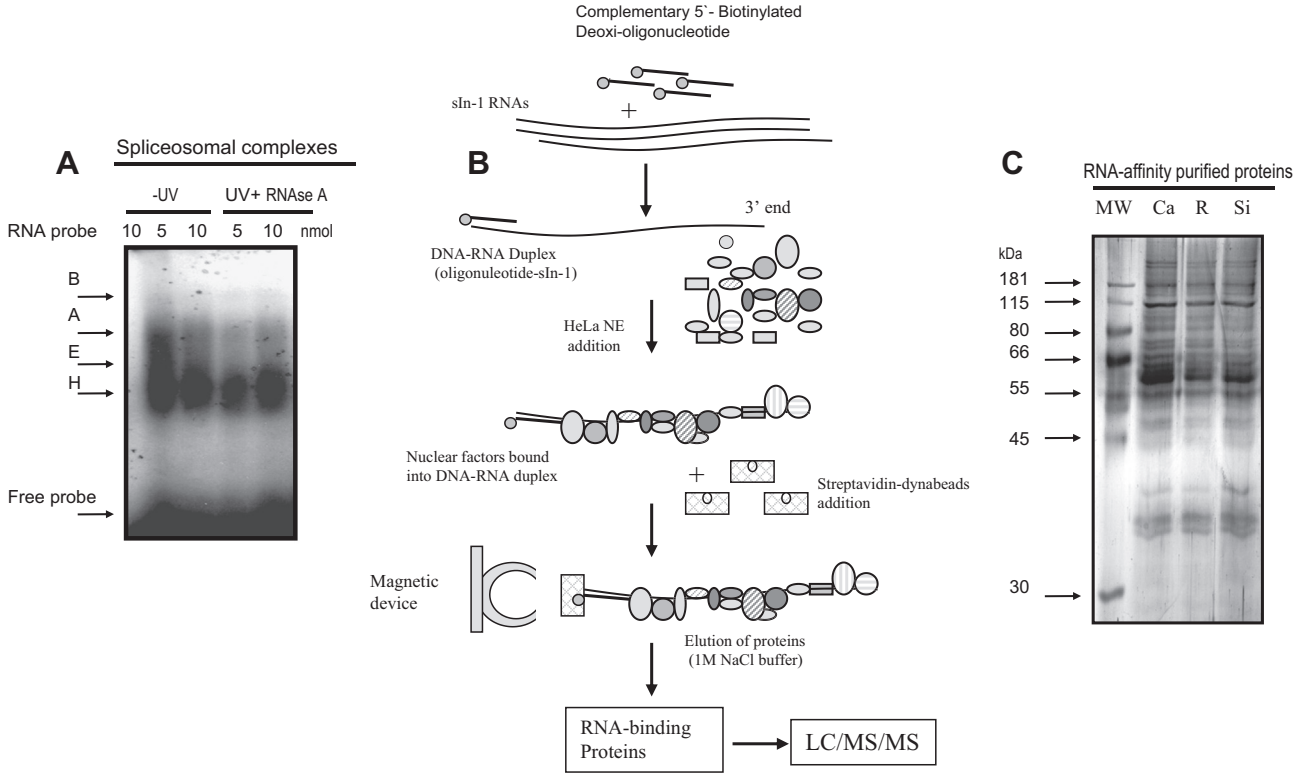

Fig. 3 – Characterization of spliceosomal complexes and purification of proteins bound to the shortest E6/E7 intron. Spliceosomal H, E, A, and B complexes were analyzed using 1.5% low-melting agarose gel and TBE buffer (A). The RNA-Affinity chromatography protocol used for purification of proteins bound to the sIn-1 is schematically represented in (B). Expression profiles of proteins eluted with RNA-Affinity chromatography by using three sIn-1 sources (C). The 3 different intron RNA probes from HPV-16 were generated by PCR by using plasmids containing the reference prototype (R) sequence or the E6/E7 sequence isolated from CaSki (C) and SiHa (Si) cells as a template.

purified proteins from three representative purification experiments is shown (Fig. 3C). Multiple proteins with molecular weights ranging from 20 to 200 kDa were purified by using this RNA affinity capture procedure. The proteins bound to the sIn-1 transcript were digested and identified in a batch using LC-MS/MS analyses. As result, more than 170 proteins were identified in at least two MS analyses; 95 of them were cognate spliceosomal factors and hnRNPs. Approximately, 40 proteins corresponded to non-snRNP splicing factors. The remaining 35 were ribosomal and cytoskeletal proteins. Only 85 of the identified factors were canonical spliceosomal proteins, which assemble during the formation of complexes A and B (Table 2). The non-snRNP proteins corresponded to RNA helicases and cognate splicing factors not previously reported in spliceosomal complexes (Table 3). All reported proteins were identified in at least two independent experiments. In addition, another identified proteins, which were not listed in Tables 2 and 3, include small and large ribosomal proteins (17 and 15 proteins, respectively), acidic 60S ribosomal proteins (P0, P1, and P2), Ran GTPase, thioredoxin, prolactin inducible protein (PIP), α- and β-actin, α- and β-tubulin, vimentin, fillaggrin, dynein, and histones (H1, H2, and H4). Most of these proteins have been previously identified by others in spliceosomal complexes, by using *in vivo* or *in vitro* models. Lastly, a few proteins of unknown spliceosomal function were also identified. The most prominent of these proteins was ashwin (C2orf49/HSPC117), which is the RNA ligase of the tRNA splicing complex. Ashwin was detected in two purification experiments.

Taken together, the results indicated that stable and reproducible protein complexes were bound to the sIn-1. The protein profile of the snRNP and non-snRNP splicing factors identified corresponded to spliceosomal A- and B-complexes. Although few of these proteins were involved in activation of the B-complex, most the specific components of the active spliceosome were not found.

4. Discussion

In this study we characterize and identified proteins that were bound to HPV-16 intron-1 (In-1) by using molecular biology, biochemistry, and proteomic tools. In-1 is unusual, because is embedded in coding sequences. Approximately, 95 of the identified proteins were canonical spliceosomal components, the other were splicing factors not previously reported as constituents of spliceosomal complexes. The HPV-16 $E6/E7^{pre\text{-}mRNA}$ is alternatively spliced and produces 4 transcripts. One of them is the unspliced pre-mRNA self, which is the most important of these mRNAs, since it is the only source of the full length E6 oncoprotein [21,25]. In-1 is constituted by one donor site and three acceptor sites, but only the first two acceptors are located in the E6 gene. Interestingly, the E6/E7 alternative

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Table 2 – The snRNP splicing factors. UniProtKB and Swiss-Prot accession numbers are given.
References are represented by superscript lowercase letters, where a refers to the detected mixed A and B spliceosomal complexes in Bennett et al. [60]; b is that detected in B complexes in Deckert et a l. [34]; c, detected in A, B, or C complexes in Herold et al. [59]; d, detected in A, B, or C complexes in Agafonov et al. [35]; e, detected in C complexes in Jurica et al. [17]; f, detected in C complexes in Yang et al. [54]; g, detected in mixed complexes in Zhou et al. [38]; h, Rasche et al. [61]; i, detected supra-spliceosomal (SS) complexes isolated in bulk in Chen et al. [33]; and j, detected in Behzadnia et al. [52]; spliceosomal complex = sp1 complex.

UniProtKB/Swiss-Prot	Protein name/short name	MW (kDa)	Spl. complex	Function
Sm proteins	hnRNP Particle components			
1. RUXG_ Human, P62308	snRNP G polypeptide/SNRPG	8.5	A [d] B [b]	Core snRNP constituent
2. SMD1_ Human, P62314	snRNP D1 polypeptide/SNRP D1	13.3	A [d] B [b]	Core snRNP constituent
3. SMD2_ Human, P62316	snRNP D2 polypeptide/SNRPD2	13.5	A [d] B [b]	Core snRNP constituent
4. SNRPD3_Human, P62318	snRNP D3 polypeptide/SMD 3	13.9	A [d] B [b] C [e]	Core snRNP constituent
5. RSMB_ Human, P14678	snRNP B and B′ polypeptide/SNRPB	24.6	A [a,d] B [a,b] C [e]	Core snRNP constituent
U1snRNP specific polypeptide				
6. RU17_ Human, P08621	U1 small nuclear ribonucleoprotein 70 kDa/snRPN70	70	Aa B a,b	U1 snRNP constituent
7. RU1A_ Human, P09012	U1 small nuclear ribonucleoprotein A/snRNP A	33	Aa B a,b C e	U1 snRNP constituent
8. RU1C_ Human, P0234	U1 smallnuclear ribonucleoprotein C/snRNP C	20	Aa B b	U1 snRNP constituent
U1snRNP associated polypeptides				
9. PRF40A_ Human, O75400	Pre-mRNA processing factor 40 homolog/FLAF1/FBP11	110	**N**	Interacts with SF1
10. DDX5_ Human, P17844	ATP dependent RNA helicase DDX5/p68	68	**N**	Pre-mRNA and mRNA binding protein
11. TCERG1_Human, O14776	Transcription elongation regulator 1/CA150/TAF2S	124	SS [i]	Interacts with SF1
U2snRNP proteins (17S)				
12. SF3A1_ Human, Q15459	Splicing factor 3a subunit 1/SAP120/SF3a120	120	A [a,d] B [a,b,c,d] C [e]	17S U2 snRNP constituent
13. SF3A2_ Human, Q15428	Splicing factor 3a subunit 2/SAP 62/SF3a66	66	A [a,d] B [a,b,c,d]	17S U2 snRNP constituent
14. SF3A3_ Human, Q12874	Splicing factor 3a subunit 3/SAP 61/SF3a60	60	A [a,d] B [a,b,c,d] C [e]	17S U2 snRNP constituent
15. SF3B1_ Human, O75533	Splicing factor 3b subunit 1/SAP155	155	A [a,d] B [a,b,c,d] C [e]	17S U2 snRNP constituent
16. SF3B2_ Human, Q13345	Splicing factor 3b subunit 2/SAP145	145	A [a,d] B [a,b,c,d] C [e]	17S U2 snRNP constituent
17. SF3B3_ Human, Q15393	Splicing factor 3b subunit 3/SAP 130	130	A [a,d] B [a,b,c,d] C [e]	17S U2 snRNP constituent
18. SF3B4_ Human, Q15427	Splicing factor 3b subunit 4/SAP 49	49	A [a,d] B [a,b,c,d]	17S U2 snRNP constituent
19. PM14_ Human, Q9Y3B4	Pre-mRNA branch site protein 14/SF3b14a	14	A [d] B [b,c,d]	17S U2 snRNP constituent
20. RU2A _ Human, PO9661	U2 small nuclear ribonucleoprotein A/SRNPA1	30	A [d] B [b,c,d]	17S U2 snRNP associated
U2snRNP (17S) related proteins				
21. SFBP _ Human, Q15637*	Mammalian branch point binding protein/mBBR/SF1	68.5	B [b,d]	17S U2 snRNP associated
22. U2AF2_ Human, P26368	Splicing factor U2AF65/	63	A [b,c]	17S U2 snRNP associated
23. PUF60 _ Human, Q9UHX1	Poly U binding splicing factor 60	60	A [d]	17S U2 snRNP associated
24. RBM17_ Human, Q96125	RNA-binding motif protein 17/SPF45	45	A [d] B [d] C [e]	17S U2 snRNP associated
25. SPF30 _ Human, O75940	Survival motor neuron domain containing 1/	26.7	A [d] B [d] C [e]	17S U2 snRNP associated
U5 specific protein				
26. PRP8_Human, Q6P2Q9	220 kDa U5 snRNP-specific protein/	220	C [d,e]	U5 snRNP constituent
27. SNRNP200_Human, O75643	U5-200 K DEXH box RNA helicase/Brr2 homolog	200	A [a] B [a] C [d, e]	U5 snRNP constituent
28. CD2BP2_Human, O95400	CD2 antigen binding protein 2/U5-52 k	52 k	B [a]	U5 snRNP constituent
29. U5S1_Human, Q15029	U5 snRNP specific protein 116 kDa/EFTUD2/hSnu114	110	A [a] B [a] C [d, e]	U5 snRNP constituent
30. SNRP40_Human, Q96D17	U5 snRNP specific protein 40 kDa/U5-40 K/WDR57	40	A [a] B [a] C [d, e]	U5 snRNP constituent
31. DDX23_Human, Q92841	ATP dependent RNA helicase DDX23/PRP28/U5-100 K	96	A [a] B [a] C [d, e]	U5 snRNP constituent
32. PRP6_ Human, O94906	Pre-mRNA processing factor 6/SFRP6/U5-102 K	107	A [a] B [a] C [d, e]	U5 snRNP constituent
33. SND1_ Human, Q7KZF4	Tudor-staphylococcal nuclease domain containing protein	100	A [f] B [a]	–
U4/U6 specific proteins				
34. PRP4 _ Human, O43172	U4/U6 snRNP 60 kDa protein/PRPF4 (WD repeat protein)	60.0	A [a] B [a] C [d]	U4/U6 snRNP constituent
35. NH2L1_ Human, P55769	tri-snRNP-associated 15.5 kDa protein/hSnu13/NHP2L1	15.5	A [d] B [d]	U4/U6 snRNP constituent

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Table 2 (*continued*)

UniProtKB/Swiss-Prot	Protein name/short name	MW (kDa)	Spl. complex	Function
U4/U6 specific proteins				
36. PP1B _ Human, P23284	Peptidyl-prolyl cis-trans isomerase A/cyclophilin A	18	N	Activation of proline containing factors
37. FKBP3_ Human, Q00688	Peptidyl-prolyl cis-trans isomerase/FKBP3	25	N	Activation of proline containing factors
U4/U6–U5 specific polypeptides				
38. SNUT1_ Human, O43290	U4/U6.U5 tri-snRNP-associated 110 k/SART1/hSnu66	110	B [b] C [d]	Tri snRNP constituent
39. SNUT2_ Human, Q53GS9	U4/U6.U5 tri-snRNP-associated 65 k/USP39/hSAD1ho	65.0	B [b] C [d]	Tri snRNP constituent
40. DHX16_ Human, O60231	Pre-mRNA-splicing factor ATP-dependent/PRP2	15.5	B [b]	Recruited to B* complex
41. SMU1 _ Human, Q2TAY7	WD40 repeat-containing protein/SMU1	57.5	B [b]	Recruited to B* complex
hPrp19/Cdc5L complex				
42. CDC5L _ Human, Q99459	CDC5 cell division 5 like	92	B [b] C [e]	prp19 complex component
43. SPF27 _ Human, O75934	Pre-mRNA splicing factor SPF27/DAM1/BCAS2	21.5	B [b] C [e]	prp19 complex component
44. PRPF19_ Human, Q9MSU4	Pre-mRNA processing factor 19 homolog/PRP19	55	B [b]	prp19 complex component
45.- BUD31_ Human, P41223	Spliceosome associate protein/Fsap17/BUD31homolog	17	N	prp19 complex component
46. CTBL1 _ Human, Q8WYA6	Beta-catenin like 1/CTNNBL1/NYD-SP19	65	B [b]	prp19 complex component
47. PQBP1_ Human, O60826	Polyglutamine binding protein 1/Npw38	37.5	B [b]	prp19 complex component
SR proteins and SR related proteins				
48. SRSF6_ Human, Q13247	Serine/arginine rich protein 55/SRRP55	55	B [b,c] B [d] C [d]	Splicing regulation
49. RSRC1_ Human, Q96IZ7	Serine rich related Protein 53/SRRP53/SRSF21	38	B [c]	Recognition of 3′ ss
50. SRRM1_ Human, Q81YB3	Serine/arginine repetitive matrix protein/SRM160	105	B [b,c] C [e]	Enhancing of splicing
Exon junction complex (EJC) and TREX COMPLEX				
51. RNSP1_ Human,Q15287	RNA binding protein S1 serine rich domain/LCD2	34	N	EJC constituent and NMD
52. DEK_ Human, P35659	DNA and RNA binding protein/DEK oncogen	43	N	EJC constituent
53. IF4A3_ Human, P38919	Eukaryotic translation initiation factor 4a/eIF4AIII	47	B [c]	RNA helicase
54. THOC4_ Human, Q86V81	THO complex subunit 4/Aly/REF	27	B [c]	Adaptor, mRNA binding protein
55. THO1_ Human, Q96FV9	THO complex subunit 1	76.6	B [c]	mRNA binding protein
56. UAP56_ Human, Q13838	Spliceosome helicase Bat 1/DDX39B	56	B [c]	Helicase, U2AF65 associated
57. PSIP1_ Human, O75475	PC4 o SFR1 interacting protein	70	N	Recognition of the BPs
58. EWS_ Human, Q01844	RNA-binding protein EWS	70	C [e]	Interacts with SF1
RNA helicases or helicase components				
59. DDX1 _ Human, Q92499	ATP dependent RNA helicase DDX1	83	SS [i]	Unwinding of dsRNA
60. DDX3X_ Human, O00571	ATP dependent RNA helicase DDX3X	73	SS [i]	Spliceosome assembly
61. DDX17_ Human, Q9BUQ8	ATP dependent RNA helicase DDX17	72	SS [i]	Pre-mRNA/RNA binding protein
62. DDX21_ Human, Q9NR30	Nucleolar RNA helicase 2/DDX21	22	B [c]	RNA binding protein
63. DDX47_ Human, Q9HOS4	ATP dependent RNA helicase DDX47	50	N	Pre-mRNA/mRNA binding protein
64. XRCC6_ Human, P12956	ATP dependent DNA helicase 2 subunit 1/Ku70	75	N	Unknown function, chromatin related
65. XRCC5_ Human, P13010	ATP dependent DNA helicase 2 subunit 1/Ku80	83	N	Unknown function, chromatin related
66. SARNP_ Human, P82979	SAP domain-containing ribonucleoprotein/	24	N	Enhances activity of DDX39A
67. DDX39A_ Human, O00148	ATP dependent RNA helicase DDX39A	49	N	RNA helicase
DNA helicase or helicase associated proteins				
68. DNAJC7_ Human, Q99615	dnaJ homolog subfamily C member7/ HSP40 member7	56	N	Chaperonin interacts with HSP90 and HSP70
69. DNAJC8_ Human, O75937	Splicing protein spf31/SPF 31/HSP40 member 8	29	SS [i]	Chaperonin, unknown function
70. DNAJC9_ Human, Q8WXX5	dnaJ homolog subfamily C member8/ HSP40 member9	30	N	Unknown

(*continued on next page*)

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Table 2 (*continued*)

UniProtKB/Swiss-Prot	Protein name/short name	MW (kDa)	Spl. complex	Function
DNA helicase or helicase associated proteins				
71. SMN1_ Human, Q16637	Survival of motor neuron 1/SMA1	32	**N**	snRNP assembly; interacts with DDX20
72. ILF2_ Human, Q12905	Interleukin enhancer binding factor 2/NF45	43	**A** [j]	Double-stranded RNABP; Interacts with ILF3
73. ILF3_ Human, Q12906	Interleukin enhancer binding factor 3/NFAR	90	**A** [j]	Interacts with Fus,DDX3X, PTB2,KU70,SMN1
74. IF16_ Human, Q16666	Interferon gamma-inducible protein 16/IFI 16	88	**N**	Interacts with SMN1
75. RUVB1_ Human, Q9Y265	RuvB like1	50	–	ATP dependent DNA helicase
76. RUVB2_ Human, Q9Y230	RuvB like2	51	–	ATP dependent DNA helicase
hnRNP family				
77. R0A1_ Human, P09651,	hnRNP A1	39	A [d] B [b,c]	Splicing regulation
78. R0AA_ Human, Q99729	hnRNP A/B	36	A [d] B [b,c]	Splicing regulation
79. R0A2_ Human, P22626	hnRNP A2/B1	38	A [d] B [b,c]	Splicing regulation
80. R0A3_ Human, P51991	hnRNP A3	40	A [d] B [b,c]	H complex
81. HNRPC_ Human, P07910	hnRNP C1/C2	38	A [d] B [b,c,d]	H complex
82. HNRNPH1_Human, P31943	hnRNPH1	50	SS [i]	Binds poly (rG)
83. HNRNPH2_Human, P55795	hnRNPH2/similar to hnRNPH1	49	**N**	Binds Poly (rG)
84. HNRPK _ Human, P61978	hnRNP K	51	B [c]	Binds poly (rC)
85. HNRPM_ Human, P52272	hnRNP M	78	A [c] B [c]	H complex
86. HNRPR_ Human, O43390	hnRNP R	71	A [d] B [c]	H complex
87. HNRPU_ Human, Q00839	hnRNP U	120	A [d]	H complex
88. R0A0_ Human, Q13151	hnRNP A0	31	A [d] B [b,c]	H complex
89. HNRPD0_ Human, Q14103	hnRNP D0	37	SS [i]	H complex
90. FUS_ Human, P35637	hnRNP/fused in sarcoma protein (TLS/FUS)	54	A [c] B [b]	Regulatory protein
91. PTBP1_ Human, P26599	hnRNP I/polypyrimidine tract binding protein 1	58	A [a] B [a] C [e]	Regulatory protein
91. PCBP2_ Human, Q15366	hnRNP E2/poly (C) binding protein/α CP2	39	B [b,c]	Binds poly (rC)
92. HNRPL_ Human, P14866	hnRNP L	64	B [c]	H complex
93. HNRDL_ Human, O14979	hnRNP D like o JKTBP1	53	**N**	H complex
94. HNRPQ_ Human, O60506	hnRNP Q	70	A [d] B [c]	H complex
95. CIRBP_ Human, Q14011	Cold inducible RNA binding protein/CIRP/hnRNPA18	31	**N**	Stress granules component

transcripts are heterogeneously expressed in tumor cells, but the cause of the heterogeneity is poorly understood [21,22]. Recognition of the In-1 splicing sequences is probably affected by the availability of a set of splicing factors, which are largely unknown. Therefore, their identification is an essential step that will shed light on the underlying mechanism producing the heterogeneity.

Spliceosomes are constituted by hundreds of proteins, which are assembled in a stepwise fashion in the pre-mRNA [26]. By using a short RNA probe and HeLa NE for *in vitro* assembly of spliceosomes; around 170 proteins were identified by LC/MS/MS, around 95 of these proteins were constituents of the A/B-complexes, since they contained U4/U5°U6 snRNP specific components. The other factors were cognate splicing factors or helicases. Noteworthy, fewer constituents of the hPrp19/Cdc5L, the exon junction complex (EJC), and the TRanscription–EXport (TREX) complex, all major components of the spliceosome catalytic core [27–29], were also identified. This was intriguing, since all preparations were made in the absence of ATP and Mg ions. Interestingly, our findings were similar in profile to those previously identified in yeast spliceosomal complexes [30,31]. The results also agreed with the number of factors isolated and identified in trypanosome [32], chicken [33] and human spliceosomes [34–38]. The function of the core constituents of the spliceosome is reasonably understood [12–14], but this is not applicable for most of the regulatory splicing factors. The function for only a few of them is known, thus, SR proteins are considered as components of splicing enhancers [39]. Intriguingly, only two SR proteins (SRRP53/SRSF21 and SRRP55/SRFS6) were identified in 2/4 preparations; similar results have been observed by other authors [35]. However, the proteins ASF/SF2 (SRSF1), SRp 20 (SRSF3) and SRp 30c (SFR9) have been reported to bind the HPV-16 early RNA. They, are part of a regulatory region, which regulates the use of the splicing acceptor 3358 (SA 3358) during the episomal life of the virus. The factor ASF/SF2 enhances splicing of the site SA 3358, while SRSF3 and SFR9, inhibit it [40,41]. In addition, other SR proteins as SRp30, SRp 55, and SRp 75 were detected in the same HPV-16 regulatory region, downstream of the SA 3358; however, their function is still not elucidated [42]. Papillomavirus nonstructural genes are expressed in early infection stages of undifferentiated keratynocites; conversely, structural genes (L1 and L2) are expressed lately in the most differentiated layer of the keratynocites. ASF/SF2 was also involved in the regulation of

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Table 3 – Non-snRNP splicing factors. UniProtKB and Swiss-Prot accession numbers are given. References are indicated in lowercase letters corresponding to those in the footnote for Table 2. Spliceosomal complex = sp1 complex.

UniProtKB/Swiss-Prot	Protein name/short name	MW (kDa)	Spl. complex	Function
Recognition of weak donor or acceptor sites				
1. TIAR_ Human, Q01085	Nucleolysin/Tia1 like protein/TIAL1/TIAR	43	N	AS factor, promotes recognition of 5′ss
2. LUC7L_ Human, Q9NQ29	Putative RNA-binding protein Luc7-like	44	(Penta snRNP)	AS factor, stabilizes U1snRNP
3. DAZ1_ Human, Q96EP5	DAz associated protein 1/DAZAP1	43	N	Unknown
4. FUBP1_ Human, Q96AE4	Far stream element (FUSE)-binding protein 1/FUBP1	68	N	Interacts with PUF60–FUSE heteromer
5. FUBP2_ Human, Q9245	Far stream element (FUSE)-binding protein2/KHSRP	73	N	Interacts with PTBP1, PTBP2 and HNRPH1
6. Sam68_ Human, Q07666	Signal transduction-associated protein 1/KHDRBS 1	68	N	AS factor, pre-mRNA/mRNA binding protein
7. ELAVL_ Human, Q15717	Elav 1 like protein/Hu antigen R/HuR	36	N	AS factor, binds AU rich elements
8. CELF1_ Human, Q92879	CUGBP/(BRUNOL) Elav like family member 1	50	N	AS factor, promotes inclusion/exclusion of exon
9. RBM15_ Human, Q96T37	Putative RNA binding protein 15/one twenty two protein	107	N	Interacts with spliceosome components
10. RBM22_ Human, Q9NW64	Functional spliceosome-associated protein 47/RBM 22	47	B [g]	Binds ISL domain of U6snRNA
12. FL2D_ Human, Q15007	Wilms tumor 1-associating/WTAP/sex letal 2D	44	–	Regulation of alternative splicing
13. SMU1_ Human, Q2TAY7	WD40 repeat containing protein/FSAP57	57	B	Splice site choice and accuracy, intron retention
NONo and NONo interacting proteins				
14. NONO_ Human, Q15233	p54[nrb]/NonO	54	C [e]	Associates with SFPQ
15. PSPC1_ Human, Q8WXF1	Paraspeckle component 1/PSPC1	59	N	NONO or SFPQ heteromer
16. SFPQ_ Human, P23246	Splicing factor proline glutamine rich	76	N	Associates with NONO
17. MATR3_ Human, P43243	Matrin 3	95	C	Interaction with SFPQ/NONO heteromer
Other RBP proteins				
18. G3P_ Human, P04406	Glyceraldehyde-3-phosphate dehydrogenase/GAPDH	36	N	Nitrosylase, unknown function
19. PDIP3_ Human, Q9BY77	Polymerase delta interacting protein 3/POLDIP3	46	N	Interacts with EJC
20. TOP1_ Human, P11387	Topoisomerase 1/topo 1	91	N	Phosphorylation of SR proteins
21. NPM_ Human, P06748	Nucleolar phosphoprotein B23 nuclephosmin/NPM1	32.5	N	Splicing negative regulator
22. APEX1_ Human, P27695	Apex endonuclease/APE1/AP lyase/APEX1	35.5	N	Associated to NPM1and pre-mRNA
23. PARP1_ Human, P09874	Poly Ado ribose polymerase1/Parp1/ARTD1	113	N	Interacts with NPM 1
24. NUCL _ Human, P19338	Nucleolin/C23	77	N	Interacts with NPM 1
25. SARNP_ Human, P82979	SAP-domain containing nucleoprotein	24	N	Interacts with DDX39B and FUS
26. IF2G _ Human, P41091	Eukaryotic translation initiation f2 sub3/EIF2S3	52	N	Unknown
27. EF1A1_ Human, P68104	Eukaryotic translation elongation factor/EEF1A1	50	–	Unknown
28. NOSIP_ Human, Q9Y314	Nitric oxide synthase-interacting protein/CGI25	33	N	Unknown
29. IFB2_ Human, P20042	Eukaryotic translation initiation f2 sub2/EIF2S2	38	–	Unknown
30. ASPVR1_ Human, Q52RT3	Retroviral-like aspartic protease 1 precursor/MUNO	37	N	Unknown
31. EXOS8_ Human, Q96B26	Exosome complex component/RRP43	30	N	CBP interacting protein
32. CAPR1_ Human, Q14444	Caprin 1	78	N	Stress granule component
33. G3BP1_ Human, I3283	Ras GTPase-activating protein	52	N	Exonuclease, stress granule component
34. CSTF1_H, Human, Q05048	Cleavage stimulation factor 1/CSTF50K	50	–	Required for 3′-end processing of mRNA
35. CPSF6_Human, Q16630	Cleavage and polyadenylation specificity factor 6	59	–	Required for 3′-end processing of mRNA.
36. CPSF5_Human, Q43809	CPSF 25 kDa Subunit/NUDT21	26	–	Recognition of two 5′-UGUA-3
37. S10A7_Human, P31151	Psoriacin 1/S100A7	11.5	–	EF hand containing protein
38. S10A8_Human, P05109	Calgranulin-A/S100A8	11	–	EF hand Ca binding protein
39. S10A9_Human, P06762	Calgranulin-B/S100A9	13.5	–	EF hand Ca binding protein
40. PDLIM7_Human, Q9NR12	Enigma/PDZ and LIM protein 7/PDLIM 7	50	N	Scaffold protein

late HPV-16 gene expression by negative regulatory element (NRE), which is thought to be pivotal in the posttranscriptional regulation of late gene expression [43]. Moreover, SRp 20 has been found to regulate the viral early-to-late switch controlling the expression of the early and late transcripts in HPV-16 and BPV-1 [42,44]. On the other hand, 20 hnRNPs were identified,

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although most of the hnRNPs are considered as constituents of the unspecific H complex [16]; they are removed by helicases in an ATP-dependent process during spliceosome assembly [37]. The function as components of splicing silencers for a few hnRNP proteins is well known [45]. During splicing of the HPV-16 E6/E7$^{pre\text{-}mRNA}$, the proteins hnRNP A1 and hnRNP A2/B1 are involved in the E6 exon exclusion; they enhances splicing between the donor 226 (SD226) and the acceptor 409 (SA 409). Interestingly, other splicing factors, namely Brr and sam68, are involved in the mechanism of the E6 exon inclusion and the mechanism is dependent of the epidermal growth factor (EGF) signaling [46]. Moreover, hnRNP A1 is also a constituent of a silencer located downstream of the SA5639, in the late HPV-16 transcript, binding sites are located in both L2 and L1 sequences; while hnRNP H binds at the L2 region [47].

Helicases are required at each of the spliceosome assembly steps [48–50]. To date, eight helicases have been suggested to play an essential role in splicing [49]. Four of these 8 helicases were bound to the sIn-1 probe (UAP56, Prp2, U5-100 K/Prp28, and U5-200/Brr2), 3 of them are U5-specific proteins. Human UAP56 was first identified as an U2AF65-associated protein, which, together with Prp5, facilitates the interchange between SF1 and U2snRNP during A-complex formation. It is also required for the recruitment of U2 snRNP to the BP during *in vitro* pre-spliceosome assembly. The U5-100 kDa helicase is involved in the U1-U6 switch at the 5′ splice site, which also requires U5-220 kDa (hPRP8) [33,49,50]. Prp 2 participates with Brr2 in the unwinding of U4/U6 duplex snRNAs, which is necessary for the annealing of U2 and U6, before the B to B* complex transition [27,50]. In addition, the GTPase U5-116 k/Snu114, which is a modulator of Brr2, was also bound to sIn-1. Lastly, another five RNA helicases were identified (DDX-1, -3X, -17, -21, and -47). However, their function during spliceosome assembly remains to be investigated. The spliceosome has dynamic composition of proteins, which changes during assembly of each of its particles [51]. Intriguingly, RNA binding proteins, such as ILF2 and ILF3, which are involved in recognition of exogenous or viral RNAs during innate immune response, were also identified. These proteins were previously purified in human A-complex preparations as NF45 and NFAR, respectively [52]. Cognate DNA helicases, such as DNAJC-7 and -9 were also identified. Noteworthy, another DNJC member was previously identified in human and chicken spliceosomes [33] therefore, the participation of these DNA helicase in spliceosome assembly could not be ruled out. Remarkably, LSm proteins were not detected. They are core constituents of the U6 snRNP particle [53]; similar results have been observed by others, in particles that clearly contain U6 snRNP [16,54]. There is evidence that LSm proteins dissociate during the formation of the B*-complexes [55]. However, in our experiments, B* spliceosomes should have not been produced since ATP was absent. One possible explanation for the lack of LSm is that the particles assembled in In-1 contained low levels of the B-complexes. Moreover, SF1 was not isolated in our assays, which further confirms that A- and B-complexes were enriched in the preparations. Furthermore, factors which are important for the second catalytic reactions (Prp16 and Prp22), or spliceosome (disassembly Prp43) [50], were not detected. However, we cannot rule out the possibility that a small amount of endogenous ATP was present. Lastly, a few factors that bind to uridine-rich sequences (or AU-rich sequences) were identified (TIA/TIAR, sam68, PUF60, HuR, WTAP, nucleolin, including the hnRNPs A2, H1, H2, C2/C2, A18, and so on). Recently, TIA 1/TIAR and Sam68 were implicated in reinforcing the recognition of weak splicing donor sites in introns, which contain downstream polypyrimidine (PPy) tracts [56,57]. A similar situation could be true for In-1, which contains PPy tracts downstream of the donor sequence [22].

Finally, our work provides the first data about the identification of factors assembled in HPV-16 intron-1. The protein profile was also similar to that of the B-like complexes, which are assembled into exons, as previously reported in human and Drosophila [26,58–60]. The profile of B-like particles differs from that of B-complexes, because it contains low levels or does not contain RBM22, CWC15, hSyf-1, and hSyf-3; in our preparations only RBM22 was detected. Cross-exon complexes, which are assembled during exon-definition, evolve into B-like complexes prior to formation of the authentic B-complexes. By contrast, cross-intron complexes evolve into the B-complexes directly from the A-complexes. This fact is relevant because HPV-16 In-1 is embedded in exonic sequences. We intend to address these aspects in future studies.

5. Conclusions

Proteomic and biochemical analysis allowed: 1) identification of 135 factors bound to the short intron of HPV-16 E6/E7$^{pre\text{-}mRNA}$. 2) Approximately 75% of these factors correspond to canonical spliceosomal components, the other 25 % are cognate splicing factors and helicases, which were not previously identified in spliceosomal preparations. 3) The factors identified suggested that the complexes were composed of a mixture of splicesomal A- and B-complexes, however, 4) the profile was also quite similar to that of the B-like complexes, which are assembled during exon-recognition.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2014.07.029.

Conflict of interest

The authors have no conflicts of interest to declare.

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