Naturally occurring heterologous *trans*-splicing of adenovirus RNA with host cellular transcripts during infection

Toyone Kikumori[1], Gilbert J. Cote*, Robert F. Gagel

Department of Endocrine Neoplasia and Hormonal Disorders, University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd, Box 435, Houston, TX 77030, USA

Received 7 May 2002; revised 13 May 2002; accepted 17 May 2002

First published online 3 June 2002

Edited by Hans-Dieter Klenk

Abstract The impact of viral infection on normal host RNA processing remains largely unexplored. We postulated that the high abundance of virally derived nuclear RNA in infected cells could impact host cell RNA splicing and viability. To test for aberrant RNA splicing we examined *trans*-splicing following infection with the replication-competent adenovirus mutant d11520 that lacks E1B 55 kDa protein. *Trans*-splicing was observed between viral RNA and several cellular precursor mRNAs, including β-actin and glyceraldehyde-3-phosphate dehydrogenase. Using a tetracycline-inducible model system simulating viral *trans*-splicing activity we observed that overexpression of a *trans*-splicing RNA specifically inhibited cell proliferation. These results demonstrate that heterologous *trans*-splicing occurs naturally during adenovirus infection and suggest that *trans*-splicing may contribute to disruption of cell function.

Key words: Heterologous *trans*-splicing; Adenovirus; Viral infection; Cell viability

1. Introduction

It is well established that the splicing of mRNA precursors is an essential step in the maturation of almost all mRNAs in higher organisms. For the most part this process occurs in *cis*, splicing together exons within a single RNA precursor. However, recently several examples of naturally occurring *trans*-splicing (the splicing of exons between two independently transcribed pre-mRNAs) of mammalian pre-mRNAs have been described [1–10]. It has been suggested that the creation of intergenic mRNA molecules through *trans*-splicing may represent an important mechanism for the creation of genetic diversity and that the process is highly regulated [11]. This ability of two independently transcribed RNA precursors to '*trans*-splice' and form a unique mRNA product also has led to the proposal that this machinery could be usurped to perform therapeutic gene correction at the RNA level. Indeed, studies by Puttaraju et al. [12] found that overexpression of a precursor *trans*-splicing molecule (PTM) that contained an RNA targeting sequence, an unpaired 3′ splice site (3′ ss) and the corrected gene sequence, could be used therapeutically to modify specific mutant RNAs. This approach has since been used both in vitro and in vivo to successfully repair mutant CFTR, Collagen 17A1 and h-*ras* mRNAs [13–16]. We used this approach in an attempt to correct mutations in mutant RET proto-oncogene RNA precursors, but those experiments had an unexpected result [17]. Overexpression of an RNA containing a single exon with an unpaired adenovirus 3′ ss resulted in widespread non-targeted *trans*-splicing and decreased cell viability [17]. This finding led us to ponder whether non-specific *trans*-splicing was unique to this particular experimental model or perhaps was a natural occurrence during the course of viral infection that might play a significant biological role.

The exact process by which adenovirus infection ultimately mediates cell death following infection remains unclear. The discovery of programmed cell death (apoptosis) and the involvement of numerous adenoviral proteins in this process have led to a renewed interest in the mechanisms involved in adenoviral-mediated cell death. There appear to be at least four adenoviral proteins involved in host cell death, E1a protein, E4ORF4, E4ORF6/7 and the adenoviral death protein (ADP) (reviewed in [18]). The E1a and E4 proteins induce cell death through the utilization of the p53-dependent and -independent apoptotic pathways, though the precise mode of action is unclear. For E4ORF4, recent studies have also shown that in addition to regulation of gene expression at the transcriptional level, E4ORF4 has the ability to modulate alternative RNA splicing [19]. E4ORF4 interacts specifically with a subset of the SR proteins, SF2/ASF and SRp30c, but not with SRp20, SC35, SRp40, SRp55 or SRp75. This interaction renders these splicing factors inactive, has a demonstrated impact on adenovirus RNA splicing, and is likely to affect host RNA splicing. Whether E4ORF4 affects *trans*-RNA splicing is unknown. The ADP gene product is an integral membrane protein that is required for efficient host cell lysis. While these viral proteins clearly play critical roles in viral-mediated cell death, other epigenetic phenomena are thought to play essential roles. For example, it has been proposed that adenovirus selectively promotes the export of viral RNAs from the nucleus, blocking the transport of normal cellular mRNAs to the cytoplasm, ultimately preventing translation of key proteins required for cell viability [20]. Our results led us to consider an additional mechanism that might contribute to viral-mediated cell death.

*Corresponding author. Fax: (1)-713-794 4065.
E-mail address: gcote@mdanderson.org (G.J. Cote).

[1] Present address: Department of Surgery II, Nagoya University School of Medicine, 65 Tsurumaicho, Showaku, Nagoya 466-8550, Japan.

Abbreviations: 3′ ss, 3′ splice site; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MLT, major late transcript; PTM, precursor *trans*-splicing molecule; RACE, rapid amplification of cDNA ends

0014-5793/02/$22.00
PII: S0014-5793(02)02878-8

The replicative phase of viral infection parallels somewhat what is observed following transfection of an expression plasmid. In both situations, there is excessive RNA accumulation in the nucleus. Hypothetically, the presence of excessive nuclear viral precursor transcripts could lead to *trans*-splicing between viral RNAs and cellular RNA precursors. Although *trans*-splicing is believed to be strictly regulated, transfection of an expression plasmid or viral infection may overwhelm the normal regulatory mechanisms [2–11]. The result would be the production of a variety of non-specific chimeric *trans*-spliced RNA products that could either directly or indirectly impact cellular function sufficiently to cause cell death. In this study, we examined if *trans*-splicing occurred between viral and host mRNA precursors during infection and what role this aberrant *trans*-splicing might play in adenovirus-mediated cell death.

2. Materials and methods

2.1. Cell lines and constructs

U251MG cells, which harbor a mutant allele of p53, were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1, v/v) with 10% fetal bovine serum (FBS). JEG-3 cells were cultured in MEM with 10% FBS. NIH 3T3 cells were cultured in DMEM with 10% FBS. Construction of PTM plasmids is described elsewhere [17]. pTRE-*myc*/*his* and pTRE-3′ ss *myc*/*his* were created by insertion of the *Bam*HI/*Pvu*II fragment of pTMΔ3′ss and pTM into the corresponding sites of pTRE (Clontech Laboratories, Palo Alto, CA, USA). Development of the tetracycline-inducible system in NIH 3T3 cells was carried out according to protocols provided by manufacturer.

2.2. Adenovirus and infection

The dl1520 is a human group C adenovirus, serotype 5 and is described elsewhere [21]. The construct used in this study is a generous gift of Dr. R. Alemany and Dr. Curiel, Unit of Gene Therapy, University of Alabama at Birmingham, AL, USA. U251MG cells were plated on a 10-cm culture dish prior to infection. The cells were infected using 100 MOI of Ad dl1520, which is replication-competent in p53 mutant cell lines as reported previously [22].

2.3. Transfection

Transfection was carried out using Gene Porter 2 reagent (Gene Therapy Systems, San Diego, CA, USA) according to protocols provided by the manufacturer.

2.4. RT-PCR and sequencing

Reverse transcription was performed using mRNA capture kit (Roche Diagnostics, Indianapolis, IN, USA) according to protocols provided by the manufacturer. A touchdown program was employed for the first round PCR. Following the initial 3 min denaturing step at 94°C, three steps consisting of 15 s for denaturing at 94°C, 30 s for annealing and 1 min for elongation at 72°C were repeated for 10 times. Annealing temperature began with 68°C and decreased 1° for each cycle. Following the touchdown phase, conventional 30-cycle PCR (15 s at 94°C, 20 s at 57°C, 1 min at 72°C) was carried out. For semi-nested PCR, 2 μl of 1/10-diluted first round PCR product was added to second PCR. PCR products (15 μl) were electrophoresed on 8% TBE polyacrylamide gels and visualized by ethidium bromide staining. Sequencing of spliced products is described elsewhere [17].

2.5. 5′ RACE

Identification of chimeric RNAs was carried out using the 5′ RACE system (rapid amplification of cDNA ends), version 2.0 (Life Technologies) according to the manufacturer's protocol with minor modifications. In brief, 5 μg of total RNA was annealed with biotin-conjugated oligo-$(dT)_{20}$ primer (5 pmol) in lysis buffer for mRNA capture kit and purified using streptavidin-coated PCR tube (Roche) (as above for RT-PCR). The cDNA synthesis and homopolymeric tailing was performed according to the protocol provided by the manufacturer. The tailed cDNAs were then amplified using the abridged anchor primer (Life Technologies) and Ad ex2-R1 primer. The first round PCR product was then amplified using nested primers (AUAP (Life technologies) and Ad ex2-R3). Prior to the second PCR step, products were digested with *Pvu*II, which is located in the major late transcript (MLT) leader exon 1, to reduce intrinsic adenoviral MLT mRNA-derived products. Following the second PCR step, PCR products were purified using the High Pure PCR Product Purification kit (Roche) and finally cloned into pGEM-Teasy plasmid (Promega). DNA sequencing used the Minx-R primer.

2.6. Metabolic labeling and immunoprecipitation

For detection of *myc*/*his*-tagged cellular proteins, NIH 3T3 (0.25×10^6 cells/well on a six-well plate) cells were transfected with pTM or pTMΔ3′ss or pVP22/*myc*-His. Transfected cells were metabolically labeled with [^{35}S]methionine (25 μCi/well) for 4 h. The labeled cells were washed and lysed in RIPA buffer containing 2 mM EDTA, 1 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride. The supernatant was cleared by incubation with protein G-Sepharose (Amersham Pharmacia Biotech, Arlington Heights, IL, USA). Cleared lysate was incubated with 1.5 μl of anti-*myc* (Invitrogen, Carlsbad, CA, USA), 5 μl of anti-actin (Santa Cruz, Santa Cruz, CA, USA) antibodies and 15 μl protein G-Sepharose in 500 μl NET-gel buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, 0.25% gelatin, and 0.02% NaN_3) overnight at 4°C. Following two washes with Nonidet P-40 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40), the pelleted antibody–antigen complex was denatured and analyzed in 16% SDS–PAGE. Radioactivity of each lane was estimated using NIH image software (ver. 1.62).

2.7. Cell proliferation assay

Ten thousand cells from each clone line (pTet-On pTRE *myc*/*his* or pTet-On pTRE 3′ ss *myc*/*his*) were plated into a 24-well tissue culture plate the day before addition of doxycycline (2 μg/ml) (day 0). The medium was replenished every 2 days. Cell number was counted using a hemocytometer. The experiment was performed in quadruplicate. Colorimetric assay (WST-1 assay system, Roche) yielded similar results.

2.8. Primers

Ad ex2-R1, TTCGGAGGCCGACGGGTTTC; Ad ex2-R3, CCGATCCAAGAGTACTGGAA; BGHrev, ACTAGAAGGCACAGTCGAGGCT; Hisrev, CAATGGTGATGGTGATGATGAC; GAPDH-F1, AGTGGATATTGTTGCCATCA; GAPDH-F2, GACCCCTTCATTGACCTC; β-actin-F1, CAACTGGGACGACATGGA; β-actin-F2, TCTGGCACCACACCTTCTAC; Minx-R, TGGAAAGACCGCGAAGAG.

3. Results and discussion

We have previously observed that overexpression of an unpaired adenovirus 3′ ss derived from exon 2 of the MLT resulted in widespread non-targeted *trans*-splicing with other RNA precursors [17]. This finding led us to question whether the same 3′ ss within the intact naturally occurring adenovirus would also be capable of *trans*-splicing during adenoviral infection. To address this question we used the well-characterized replication-competent adenovirus strain Ad dl1520 [22,23]. This specific virus has been widely employed as an oncolytic agent because it is only capable of lytic replication in p53-deficient cell lines due to the lack of E1B-55 protein [22]. Total RNA was isolated during productive infection (48 h post infection) to determine whether targeted *trans*-splicing could be detected between two common genes (glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin) and the adenovirus type 5 MLT leader exon 2. An initial RT-PCR reaction was performed using exon-specific primers followed by 30 cycles of semi-nested PCR in order to reduce RT-PCR background (Fig. 1A). Very little product was observed after the first round of RT-PCR suggesting that *trans*-

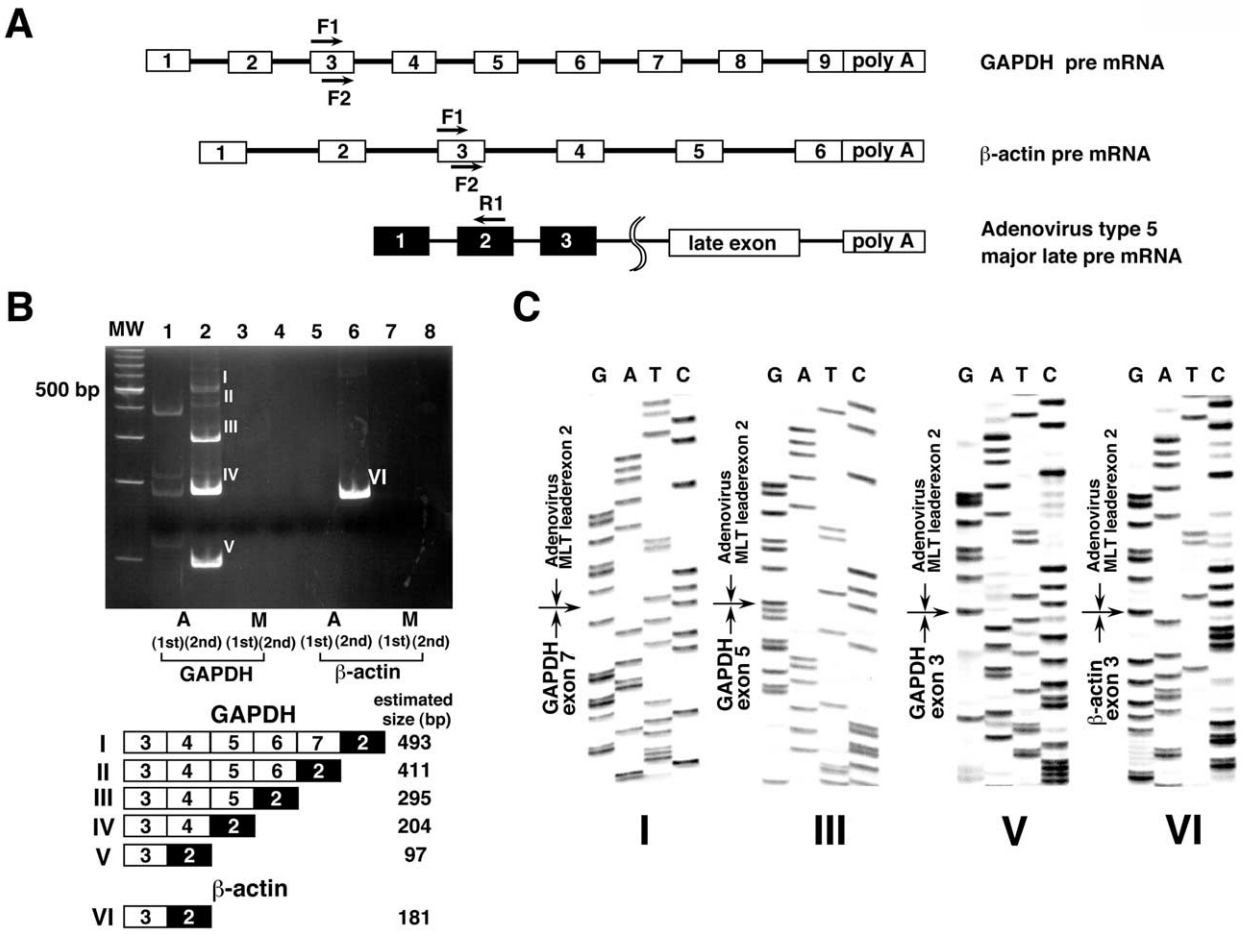
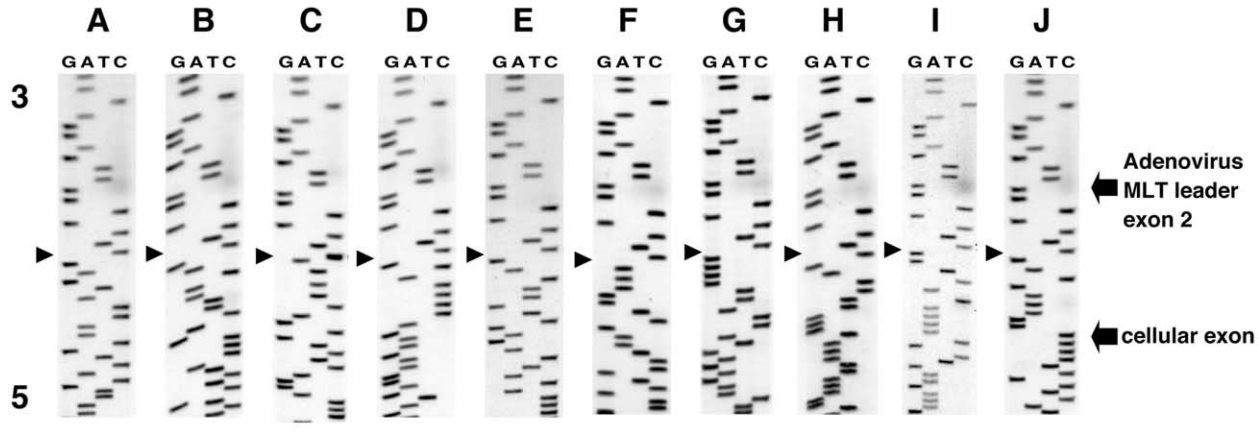


Fig. 1. *Trans*-splicing is associated with adenovirus infection. A: Structure of GAPDH, β-actin and adenovirus type 5 MLT precursor mRNAs showing the relative position of the primers used for amplification of *trans*-spliced products (figure is not to scale). B: RT-PCR of RNA from the cells infected with adenovirus. Roman numerals indicate presumed chimeric products schematically represented. A, adenovirus-infected cells; M, mock-infected cells; 1st, first round PCR; 2nd, semi-nested PCR. MW, 100-bp DNA ladder. C: Direct sequencing of *trans*-spliced products. Arrows indicate the splice junctions between GAPDH or β-actin exons and adenovirus MLT leader exon 2. Roman numerals correspond to those in panel B.

splicing products did not exist at high abundance (Fig. 1B). However, following the second semi-nested PCR we were able to detect several RT-PCR bands that correspond to predicted *trans*-splicing products (multiple introns of the GAPDH gene were involved). The presence of these bands was dependent on viral infection and not observed in mock-infected cells (Fig. 1B). Sequencing of the product bands confirmed accurate use of existing splice sites, suggesting that these products were generated through a *trans*-splicing mechanism (Fig. 1C). Integration of adenovirus into the host genome, another mechanism for producing the observed products, has not been reported for productive viral infection although a few examples have been described for non-productive infection [24,25]. To exclude this possibility, we performed PCR using genomic DNA from the virally infected cells. No PCR products were detected, confirming our contention that these RNAs were generated through a *trans*-splicing mechanism (data not shown).

This finding was unexpected and raised several important questions. The first was 'How widespread is this phenomenon?' Our initial study only examined *trans*-splicing between a single adenovirus exon and two specific genes. To explore the extent to which cellular pre-mRNA is captured by adenovirus pre-mRNA through a *trans*-splicing mechanism, we performed 5′ RACE with RNA from the infected cells using downstream primers located in the MLT leader exon 2. To reduce the presence of MLT *cis*-spliced products the 5′ RACE cDNA was digested with *Pvu*II following the first round of PCR. This enzyme restricts a site located in the adenovirus MLT exon 1. Sequencing of 10 clones indicated that a broad spectrum of cellular pre-mRNAs undergo *trans*-splicing during productive infection (Fig. 2). Two common features of sequenced products were observed: *trans*-splicing occurred in a large intron (most are >20 kb); and splicing occurred in the first intron or in an intron close to the 5′ end of the pre-mRNA. The former implies that genes with large introns may be more susceptible to *trans*-splicing. The latter feature may be attributed to technical restriction of 5′ RACE (where shorter products are preferably amplified).

While our 5′ RACE experiment gave some idea of the diversity of precursor mRNAs subject to *trans*-splicing, it did not address the overall importance of *trans*-spliced transcripts generated by adenoviral infection. None of the chimeric gene transcripts identified seem to be targets that would be critical to cellular proliferation. To show *trans*-splicing is an important process in this context, we developed a construct to test



Identity	GenBank Accession Number cDNA	Genomic
A: interferon-inducible gene (IFI)-54K, 1st coding exon	X07557	AL353751
B: v-abl Aberlson murine leukemia viral proto-oncogene homolog 2 transcript variant a (Exon 1A)	NM005158	AC24503
C: T-plastin exon 1	M34427	AF007295
D: TAFII 18 mRNA for TFIID, 1st coding exon	X84003	AQ512573
E: zinc finger protein 41 exon 3	X60155	AL160281
F: armadillo protein P0071 5 UTR	X81889	AC008070
G: GTP-binding protein (RAB5) 1st coding exon	M28215	AC005832
H: Homo sapiens HDCMC28P protein	AF068285	AL161659
I: unknown	AL512685	AF165926
J: unknown	W96564	AC005476

Fig. 2. A broad spectrum of cellular genes undergo *trans*-splicing. The sequencing of several clones derived by 5′ RACE is shown. Arrowheads indicate the splice junction between the last nucleotide of a cellular exon involved and the first nucleotide of adenovirus MLT leader exon 2. The identities of *trans*-spliced genes are indicated at the bottom of the figure.

the hypothesis that *trans*-splicing alters cellular functions. A PTM was generated using the 3′ ss sequence of adenovirus type 5 MLT intron 1 followed by human c-*myc* epitope (30 nt), polyhistidine molecular tag (18 nt) (*myc*/*his*) and bovine growth hormone polyadenylation signal (Fig. 3A) [17]. This construct can only produce a translatable mRNA by *trans*-splicing. A control vector in which the 3′ ss was removed was also generated (pTMΔ3′ss). The PTM was transfected into JEG-3 (human choriocarcinoma cell line) by lipofection. This experiment showed *trans*-splicing between the GAPDH and β-actin, analogous to that seen for adenovirus infection (compare Figs. 1B and 3B). The authenticity of these chimeric transcripts was verified by direct sequencing of PCR products (data not shown). These chimeric transcripts were considered to be generated through *trans*-splicing since no equivalent products were detected from genomic DNA of the transfected cells and transfection of pTMΔ3′ss generated no equivalent products (data not shown).

To provide some estimate of the extent to which cellular mRNA undergoes *trans*-splicing, we used the human c-*myc* epitope to immunoprecipitate chimeric proteins (using an anti-human c-*myc* antibody). Use of a mouse cell line (NIH 3T3) avoided cross-reaction and immunoprecipitation of endogenous c-*myc*. Cells were transfected with pTMΔ3′ss, pTM or pVP22/*myc*-His (Invitrogen) which generates *myc*/*his*-tagged VP22 protein (45 kDa) as a positive control for the anti-*myc* antibody. Following transfection, cells were metabolically labeled with [^{35}S]methionine to examine new protein synthesis. Immunoprecipitation using anti-*myc* and anti-actin (for an internal control) antibodies is shown in Fig. 4A. The lysate of the cells transfected with pVP22/*myc*-His clearly demonstrated the presence of *myc*/*his*-tagged VP22 protein. A comparison of precipitable protein observed in cells transfected with pTMΔ3′ss versus pTM demonstrated the emergence of innumerable c-*myc*-tagged protein bands, considered to be chimeric proteins generated from *trans*-splicing of cellular pre-mRNA and the PTM. The overall level of tagged proteins detected was three-fold greater than observed in control cells, which is even more striking when one considers that only *trans*-splicing events that provide an in-frame start codon can be detected.

The balanced spectrum of protein sizes observed in the immunoprecipitation experiment and the range of RACE products cloned during adenoviral infection suggest that *trans*-splicing mediated from this adenovirus splice site is non-specific and prevalent, though this does not rule out the possibility that specific regulatory chimeric RNAs are produced. This raises the obvious question of whether unregulated *trans*-splicing might be deleterious to normal cellular function. That this was the case was demonstrated in two ways. First, we attempted to create stable clones from PTM-transfected cells and were unsuccessful despite several attempts, whereas stable clones were readily developed from the control vector (pTMΔ3′ss). This result suggests that the disadvantageous effects of PTM expression eliminate clones under selection pressure. Second, since expression of PTM is presumed to be

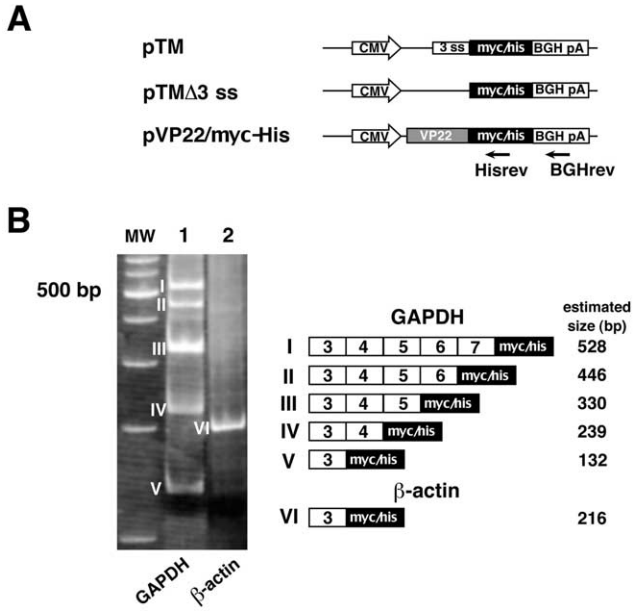


Fig. 3. A PTM simulates the *trans*-splicing activity of adenovirus. A: Schematic representation of vectors used (CMV, cytomegalovirus immediate early promoter and enhancer; BGHpA, bovine growth hormone polyadenylation signal). B: RT-PCR of RNA from JEG-3 cells transfected with pTM expression vector. Roman numerals indicate presumed chimeric products schematically represented.

detrimental to cell viability, we created stable clones capable of tetracycline-inducible expression of PTM (pTRE-3′ ss *myc/his*) or control RNA (pTRE *myc/his*). The constructs used to create these clones are identical to the plasmids shown in Fig. 3A except that the CMV promoter has been replaced with a tetracycline-responsive promoter (TRE). Expression of the *myc/his* or 3′ ss *myc/his* sequence was up-regulated by the addition of doxycycline (Fig. 4B, inset). Furthermore, induction of the PTM by doxycycline was capable of a modest, but significant inhibition cell growth, whereas no effect on growth was observed for the control cells (Fig. 4).

Our findings indicate clearly that *trans*-splicing between cellular host pre-mRNAs and adenovirus pre-mRNA occurs during the natural course of infection. This *trans*-splicing is promiscuous and occurs at multiple splice sites within a given gene transcript. To the best of our knowledge, this is the first example of naturally occurring heterologous *trans*-splicing between viral and cellular host RNA transcripts. Our finding is not entirely unexpected given that the artificial overexpression of foreign RNA precursors by transfection has clearly been demonstrated to cause *trans*-splicing [12–17]. Indeed, recent studies by Caudevilla et al. [26] have shown that plasmids encoding the HIV-nef viral transcript when expressed in CV-1 cells is capable of *trans*-splicing with host cell RNA precursors, lending support to the idea that *trans*-splicing occurs naturally during viral infection. However, in this report it was the viral RNA transcript that was targeted by *trans*-splicing mediated through the 3′ ss of host cell precursor RNAs. What is unclear is whether the targeted HIV-nef 5′ splice sites and the 3′ ss sequence of adenovirus type 5 MLT intron 1 represent unique cases where these splice sites are more prone to *trans*-splicing. For the carnitine octanoyltransferase gene, a specific purine-rich exonic enhancer (GAAGAAG) is responsible for promotion of *trans*-splicing [27]. Similar purine-rich sequences exist throughout the adenovirus genome including MLT exon 2, however we have no experimental evidence to suggest that these sequences function to enhance *trans*-splicing of adenoviral transcripts with host cell RNAs. In addition, the effects of E4ORF4 to inhibit SR protein activity, which are mediators of many splicing enhancer actions, at least argues against an SF2/ASF- or SRp30-mediated enhancement of *trans*-splicing [19].

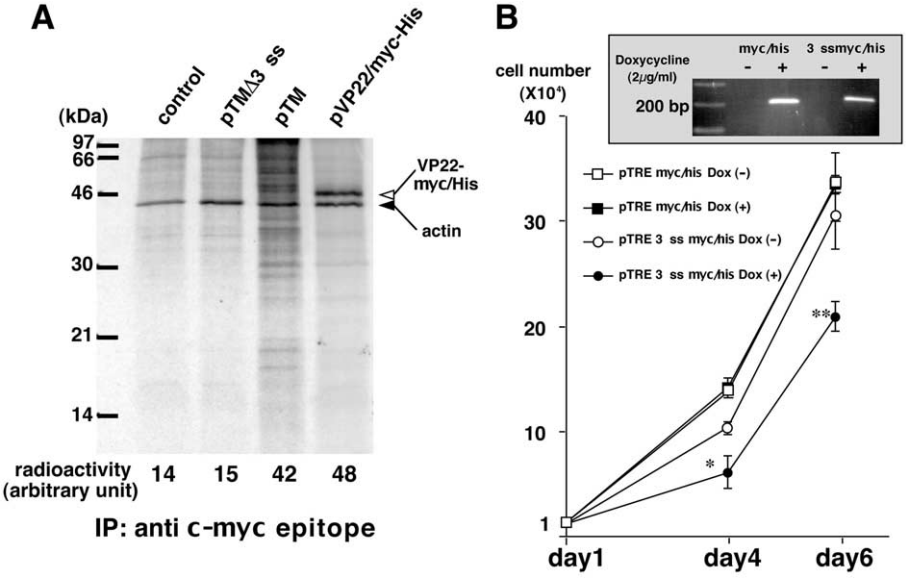


Fig. 4. *Trans*-splicing to cellular RNA creates a spectrum of protein products and alters cell proliferation. A: Transfection of pTM induced the generation of chimeric protein products detectable by anti-c-*myc* immunoprecipitation. The plasmids used for transfection are indicated at the top of each lane. Relative counts for the entire lane are shown. B: Cell proliferation of NIH 3T3 clones in which expression of pTM is inducible by addition of doxycycline (tetracycline-derivative). $*P < 0.05$; $**P < 0.01$ against uninduced control. Inset, induced expression of *myc/his* or 3′ ss *myc/his* sequence with doxycycline. RNA from the cells of each clone was subjected to RT-PCR using expression unit-specific primers (25 cycles).

In addition, we have demonstrated that *trans*-splicing between abundantly expressed vector-derived RNAs and endogenous pre-mRNA inhibits cell growth. While these findings are provocative because they suggest a potential epigenetic mechanism whereby aberrant *trans*-RNA splicing may disrupt cell proliferation, they do not address if *trans*-splicing plays a specific role in adenoviral-mediated host cell death. However, we perceive two potential mechanisms by which *trans*-splicing could act to impact cell viability. In the first *trans*-splicing could result in the production of a specific and unique product that profoundly affects cell growth or death. For example, targeted *trans*-splicing of any of several anti-apoptotic genes would be predicted to enhance cell death. Alternatively, the phenomena may be non-specific. In this case, the broad-based disruption of *cis*-splicing would be expected to cause a decreased production of critical cellular proteins responsible for host cell growth. That either mechanism could play a distinct role in adenovirus-mediated disruption of cellular function provides an intriguing area for additional investigation.

Acknowledgements: This work was supported by NIH Grant CA67946 (G.J.C.) and the John Ball and James Meszaros Research Funds (R.F.G.). We thank Juan Fueyo for his generous gift of RNA from adenovirus-infected cells and helpful discussions, and Miles Wilkinson for his critical comments.

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