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Mechanism of RNA synthesis initiation by the vesicular stomatitis virus polymerase.

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 17 August 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. Please let me first apologise for the delay in getting back to you with a decision: this is due to a very high submission rate at present, as well as the absence of several editors from the office. I have now had a chance to read your study carefully and to discuss it with some of my colleagues and I am sorry to say that we cannot offer to publish it.

We appreciate that you have analysed the mechanism by which the VSV RdRP, the L protein, initiates RNA polymerisation. You demonstrate that L can initiate synthesis on naked RNA, in the absence of the nucleocapsid protein that normally encapsulates the RNA and the P phosphoprotein that links L to N. Initiation is via a *de novo* mechanism, as predicted, and you determine the sequence requirements on the 3' end of the template RNA. You further show that P is a processivity factor, although full processivity must require other components. While we do recognise that this work will be of interest to the immediate field, I am afraid we find the overall conceptual advance - from the perspective of the broad readership of the EMBO Journal - to be somewhat limited: it is already known that other negative-strand RNA viruses initiate synthesis *de novo* via a templated mechanism, and your recent work has shown that the RdRP of other viruses can recognise its template in the absence of cofactors. Consequently, we can not offer to consider your work further for publication in the EMBO Journal, and suggest it would be better suited to the more specialised virology literature.

Please note that we publish only a small percentage of the many manuscripts that we receive at the EMBO Journal, and that we can therefore subject to external review only those submissions that

have a good chance of timely acceptance. I am sorry to disappoint you on this occasion, but I hope that this negative decision will not prevent you from considering the EMBO Journal for publication of future studies.

Yours sincerely,

Editor
The EMBO Journal

Rebuttal 17 August 2011

Thank-you for your e-mail outlining your decision below. It seems that I did not clearly emphasize why our study would appeal to a broad and diverse readership and I would like you to consult someone in the field of negative-strand RNA viruses about our work, for the following reasons.

1: Although synthesis on naked RNA has been shown for influenza and Machupo viruses those viruses have segmented negative strand (SNS) RNA virus genomes. The nucleocapsid templates of SNS RNA viruses are fundamentally distinct to the nonsegmented negative stand (NNS) RNA viruses in that the segmented RNA virus genomes are loosely associated with the N protein, not completely sequestered as shown by the atomic structures of the N-RNA templates of rhabdo, paramyxo and bornaviruses. The strategy of RNA synthesis is completely different too - in that NNS RNA viruses sequentially transcribe mRNA from the same template, whereas the SNS RNA viruses only transcribe single mRNAs from their templates.

2: It has been dogma in virology for 41 years that RNA synthesis does not work on the naked RNA of nonsegmented negative stand RNA viruses. Prior to our study, the only way to study RNA synthesis *in vitro* for this entire order of viruses was to purify virus particles or the N-RNA template from virus. Since, with the exception of VSV, none of the viruses grow to a reasonable titer it has not been possible to study RNA synthesis *in vitro* and consequently perform meaningful mechanistic studies except with VSV. This severely limits the study of the process of RNA synthesis, our knowledge of the mechanism, and importantly the major quest for identification of inhibitors of L - an objective of several of the worlds leading pharmaceutical companies (Roche, Novartis, Sanofi etc)

3: Our system is so robust and versatile that it has already been translated into the study of other NNS L proteins - for which since the dogma dictated the experiment would not work, people have not attempted. For example, we and a colleague have now successfully reconstituted RNA synthesis for respiratory syncytial virus *in vitro*. Indeed, this approach will work for any L protein that can be expressed and purified.

4: Different NNS RNA viruses utilize subsets of L cofactors - although they all employ a P protein. It has never been possible to define the role of P in regulating L activity owing to the obligate requirement of N on the template. This approach will also - for the first time - allow definition of the mechanism by which other L cofactors function.

5: The group of viruses that this work can be extended to, include some of the most significant human, animal and plant pathogens extant - many of which there are no vaccines, and for none of which there are antiviral drugs. Among this list are the 100% fatal rabies virus, the highly lethal Ebola and Marburg virus, the highly contagious measles virus, as well as mumps and respiratory syncytial virus. Our study promises to revolutionize the hunt for inhibitors of the L protein polymerases for these viruses, one of the major targets for antiviral drug development. The L protein is a highly attractive target since it is a single protein that synthesizes RNA as well as caps, methylates and polyadenylates the mRNA.

In light of these points, I would ask that you consult with someone in the field to determine their impression of our work.

Editorial Correspondence 29 August 2011

I have now discussed your manuscript with an expert editorial advisor, and we have decided to send your manuscript out for in-depth review. We have already made your submission a formal appeal, so you don't need to do anything. We will be in touch again once we have received reports on your study and are in a position to make a decision, or to let you know if there are any unforeseen delays.

Yours sincerely,

Editor
The EMBO Journal

2nd Editorial Decision 27 September 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three reviewers express interest in your work, although all also raise a number of concerns that would need to be addressed by a major revision of the manuscript, before we could consider publication here. Their reports are explicit, so I need not go into detail here, although I would just highlight a couple of the most critical points:

- Referee 1 (point 3) notes that you have not examined the effect of N protein on L-mediated RNA synthesis. I would strongly encourage you to follow the suggestion made here and assay the role of N.
- Referee 2 (throughout) requests sequence data to definitively demonstrate the initiation site for L. I agree with the referee that this would be important information.

Should you be able to address the various concerns raised, I would like to invite you to submit a revised version of the manuscript. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEEE REPORTS:

Referee #1:

This work by Morin and colleagues examines the minimal requirements for initiation of RNA synthesis by the RNA dependent RNA polymerases (RDRP) (L protein) of VSV, the prototypic non-

segmented negative-strand RNA genome (NNS).
Most negative strand (NS) RNA viruses, including VSV, encode for a large (ca 200 kDa) polypeptide member to the L protein family that contains conserved domains and residues characteristic of RDRP. For many NS RNA viruses overwhelming evidence indicates that L is capable of direct different functions required for RNA replication and synthesis of viral mRNAs. Both *in vitro* and cell-based studies have shown that efficient VSV RNA replication and gene transcription requires, in addition to L, the polymerase cofactor P protein and the nucleoprotein (N) required to generated the encapsidated genome RNA that serves as polymerase template. However the identification of the minimal viral components required for initiation of RNA synthesis and the underlying mechanisms has not been elucidated.

In this work the authors have demonstrated that the minimal functional unit for initiation of VSV RNA synthesis comprises a genome RNA template and the L protein, whereas P enhances both the initiation and processivity of L. In addition, the authors have provided convincing evidence that the first two positions in the template (3'-UG-5') play a critical role in initiation of RNA synthesis, whereas positions 3 to 6 within the template modulate the efficiency of this process. The authors also showed that VSV L polymerase can recognize the 3'-UG-5' specific initiation signal when internally located but the efficiency of initiation is progressively diminished as the initiating nucleotide is located further away from the 3'-end of the template RNA. Using this *in vitro* assay of initiation of RNA synthesis by the VSV L polymerase, the authors presented evidence that the nucleoside analog ribavirin, known to have a broad-range antiviral activity against a variety of viruses, can be incorporated by VSV L into the nascent RNA, which resulted in inhibition of RNA elongation thus providing an additional, among several others previously documented, mechanism of action by which ribavirin can exert its antiviral activity.

The paper is well written and the results have been clearly presented and nicely illustrated. The data presented, for the most, support well the main conclusions reached by the authors. This work should be of interest in general to those interested in the mechanisms by which L proteins recognize their templates and initiate RNA synthesis. The reported findings open also some novel interesting possibilities for the identification of molecules capable of inhibiting initiation of RNA synthesis by viral L polymerases. There are several aspects of the paper that could benefit from some additional clarifications (see below specific comments).

Specific Comments:

1) Studies showing the effect of P on initiation and processivity of RNA synthesis by VSV L would benefit perhaps by the incorporation of a control with P mutant known to lack the ability to interact with L. Likewise, results shown in Fig 5 A indicate that bacterially expressed P, which is expected to be non-phosphorylated, seems to enhance RNA synthesis efficiently, whereas previously published work has documented the requirement of P phosphorylation for efficient viral RNA synthesis and growth in infected cells. This should be discussed in more detail by the authors.

2) Results shown in Fig 6 provide convincing evidence that incorporation of ribavirin by VSV L into the nascent RNA results in inhibition of RNA elongation. To some extent this result appears quite different compared to findings reported for the polymerase of the picornavirus poliovirus. Would this mean that RDRP of positive and NS RNA viruses have intrinsic different properties regarding their ability to use ribavirin as a substrate and template? In addition, the data shown in Fig 6 would suggest that at least for VSV, and perhaps the L polymerases from other NS RNA viruses, the antiviral activity of ribavirin is mediated by its ability to inhibit RNA elongation. This should be discussed in the context of a proposed mechanism of action for ribavirin based on its potential mutagenic activity.

3) In the discussion section the authors proposed, based on their results, that N and P play regulatory roles to ensure authentic copying. However, the present work did not include per se experiments that would allow the authors to assess the role of N. In this regard, it would have been interesting to incorporate experiments where the authors examined, in addition to P, the effect of N on initiation and processivity of RNA synthesis by the VSV L polymerase.

4) In some instances in the discussion section the authors have generalized their conclusions, based on their findings with VSV L, to the L proteins in general of all NNS RNA viruses, whereas there is

some evidence suggesting that L polymerases from different NNS RNA viruses may have some differences in terms of their template requirements. Likewise the discussion about the mechanisms by which L may displace N from the template, required for L-mediated RNA synthesis, assumes that L would copy the RNA template as a monomer, but there is evidence suggesting that for at least for some NNS RNA viruses the functional polymerase requires some degree of L oligomerization, which should be further discussed.

5) I think that the discussion section could be shortened without impacting its scientific content, as it contains some redundant information already presented in the results section of the paper.

Referee #2:

This contribution to our understanding of initiation of synthesis by the VSV L polymerase breaks new ground, as this is the first report claiming that some synthesis, albeit modest, can take place with an RNA template that is not encapsidated by the viral nucleoprotein. Morin et al., show that L protein only synthesizes short products from a 19 nt long template RNA that encodes the 3' end of the viral genome. This is clearly not what the polymerase does in the natural context of VSV infection but the findings do inform our understanding of the catalytic mechanism and what L can do on its own. The paper is well written and the data are well presented. The authors' main conclusion however, i.e., that L can in fact initiate templated synthesis from the 3' end residue of the template, lacks firm support. This deficiency might well be resolved by providing sequence data of the main products, and establishing whether L can synthesize the first phosphodiester bond (AC dinucleotide) independently of template. The latter could explain why recent findings using a related virus (RSV) strongly suggest that L initiates synthesis in a non-templated fashion. The authors should address the following points.

1.Fig. 1 - The gamma A-labeled products were synthesized under limiting ATP conditions which could explain why the band pattern is very different from the alpha-G products even when accounting for the two G residues. But the band pattern of alpha-G products at 1.5 mM ATP differs substantially between Fig. 1A, Fig. 1B and Fig. 2B. Even putting aside this variation, the authors should determine the actual sequence of the major product bands before concluding that initiation must occur at the very 3' end of the template, especially since early studies using the encapsidated template showed strong evidence of internal initiations *in vitro*.

2.Fig. 1 - It is curious that the observed synthesis required high concentrations of both ATP and CTP while earlier studies using encapsidated templates clearly established that this requirement pertained to ATP only. Do the authors have an explanation for this?

3.The gel analyses shown in the various figures do not identify dinucleotides. Given the recent findings showing that the RSV polymerase can correctly initiate replicative synthesis *in vivo* on genome templates lacking the 3' end residue, or on antigenome templates lacking the first two residues, the authors' should exclude the possibility that L protein only can catalyze synthesis of the AC dinucleotide (and possibly other dinucleotides) in the absence of template ("preloaded" L). If this were the case, the authors' conclusions would need revision.

4.Figs. 2 and 3 - The inhibitory effects of base substitutions at the first two positions of the template (Fig. 2), as well as the observed synthesis when appending UG or UGC at the 3' end of a random template, are consistent with L initiating at the 3' end and the high concentration requirements for the complementary nucleotides but do not exclude a "preloaded" L initiating templated synthesis subsequent to catalyzing dinucleotide formation.

5.Fig. 4 - The results of adding an extra one, two, or three nucleotides at the 3' end of the template are difficult to interpret. The authors claim this reflects internal initiation at the proper U residue corresponding the virus genome 3' end, but this also emphasizes that L can easily start internally and, absent sequence information of the product bands, where initiation occurs remains unclear.

Referee #3:

In their paper, Morin et al. have characterized the mechanism of RNA synthesis initiation by the vesicular stomatitis virus (VSV) polymerase (L).
For this, they have taken advantage of their ability to purify a His-tagged recombinant L protein expressed in SF21 cells to establish a very simple *in vitro* system. In this system, they only incubate naked RNA with L polymerase. This differs from previous systems to study RNA synthesis *in vitro* for nonsegmented negative-strand RNA viruses, which are limited by the necessity to purify the N-RNA template from viral particles or from cells. Particularly, with these systems, the influence of the template sequence cannot be analyzed.
Here, they demonstrate that vesicular stomatitis virus L initiates synthesis via a *de novo* mechanism that is independent of N or P and needs a high concentration of the first two nucleotides and specific template requirements. They also show that presence of P enhances processivity. Finally, they use their system to characterize the mechanism of action of ribavirin demonstrating that their experimental system represents a powerful method for understanding how inhibitors of RNA synthesis function.
In general, the paper is well written, easy to read and the experimental data are convincing.
Nevertheless, I have some remarks that have to be addressed by the authors:

Major points:

1) In figures 1A and 4B, the migration profiles are very different when the neo-synthesized RNA is labeled with [32P] ATP or with [32P] GTP. They should look the same (as it is the case in figure 2B when the neo-synthesized RNA are labeled with [32P] GTP or with [32P] GTP).

2) As my previous remark raises some question about the reproducibility of the assay, I would like to see the error bars on figures 1C, 2C and 6C.

3) The authors indicate that the reactions mixture were incubated at 30ºC for 3 hours in presence of L. I would like to see some kinetic datas that justify this reaction time (and may be a comparison of these kinetics with those obtained in the previous experimental systems using an N-RNA template)

Minor points:
1) In the discussion (p14, line 257), the authors have written "Since the L-P complex itself is not fully processive on naked RNA, our findings suggest that the template associated N may also represent an important processivity factor for the RdRP". In absence of any experiment performed with N (i.e. using an N-RNA template), I would avoid to write this sentence. Processivity might be as well influenced by local nucleotide concentration and by any cellular co-factors (and even by the phosphorylation state of P).

1st Revision - Authors' Response 18 November 2011

Referee #1:

This work by Morin and colleagues examines the minimal requirements for initiation of RNA synthesis by the RNA dependent RNA polymerases (RDRP) (L protein) of VSV, the prototypic non-segmented negative-strand RNA genome (NNS). Most negative strand (NS) RNA viruses, including VSV, encode for a large (ca 200 kDa) polypeptide member to the L protein family that contains conserved domains and residues characteristic of RDRP. For many NS RNA viruses overwhelming evidence indicates that L is capable of direct different functions required for RNA replication and synthesis of viral mRNAs. Both *in vitro* and cell-based studies have shown that efficient VSV RNA replication and gene transcription requires, in addition to L, the polymerase cofactor P protein and the nucleoprotein (N) required to generated the encapsidated genome RNA that serves as polymerase template. However the identification of the minimal viral components required for initiation of RNA synthesis and the underlying mechanisms has not been elucidated.

In this work the authors have demonstrated that the minimal functional unit for initiation of VSV RNA synthesis comprises a genome RNA template and the L protein, whereas P enhances both the initiation and processivity of L. In addition, the authors have provided convincing evidence that the first two positions in the template (3'-UG-5') play a critical role in initiation of RNA synthesis, whereas positions 3 to 6 within the template modulate the efficiency of this process. The authors also showed that VSV L polymerase can recognize the 3'-UG-5' specific initiation signal when internally located but the efficiency of initiation is progressively diminished as the initiating nucleotide is located further away from the 3'-end of the template RNA. Using this *in vitro* assay of initiation of RNA synthesis by the VSV L polymerase, the authors presented evidence that the nucleoside analog ribavirin, known to have a broad-range antiviral activity against a variety of viruses, can be incorporated by VSV L into the nascent RNA, which resulted in inhibition of RNA elongation thus providing an additional, among several others previously documented, mechanism of action by which ribavirin can exert its antiviral activity.

The paper is well written and the results have been clearly presented and nicely illustrated. The data presented, for the most, support well the main conclusions reached by the authors. This work should be of interest in general to those interested in the mechanisms by which L proteins recognize their templates and initiate RNA synthesis. The reported findings open also some novel interesting possibilities for the identification of molecules capable of inhibiting initiation of RNA synthesis by viral L polymerases. There are several aspects of the paper that could benefit from some additional clarifications (see below specific comments).

We thank the reviewer for their enthusiasm and support comments of our work.

Specific Comments:

1) Studies showing the effect of P on initiation and processivity of RNA synthesis by VSV L would benefit perhaps by the incorporation of a control with P mutant known to lack the ability to interact with L. Likewise, results shown in Fig 5 A indicate that bacterially expressed P, which is expected to be non-phosphorylated, seems to enhance RNA synthesis efficiently, whereas previously published work has documented the requirement of P phosphorylation for efficient viral RNA synthesis and growth in infected cells. This should be discussed in more detail by the authors.

As requested by the reviewer, we now include a control of a P-mutant known to lack the ability to associate with the L protein (PΔ106) by deletion of the N-terminal 106 amino acids (Figure 5A and lines 209-214). As expected, reactions containing this fragment of P behave identically to those lacking any P protein.

The reviewer also requested some discussion about the phosphorylation status of the P protein. We consistently find that P expressed and purified from Escherichia coli, and thus lacking such post-translational modifications, is equally supportive of RNA synthesis in our in vitro assays. We agree with the reviewer that prior work has indicated that phosphorylation of P is essential for transcription and virus growth in vivo. However, our work does not reveal a requirement for P phosphorylation, consequently, we are left to only speculate about the function of P phosphorylation which seems beyond the scope of this study. We discuss this briefly in the text (lines 341-347).

2) Results shown in Fig 6 provide convincing evidence that incorporation of ribavirin by VSV L into the nascent RNA results in inhibition of RNA elongation. To some extent this result appears quite different compared to findings reported for the polymerase of the picornavirus poliovirus. Would this mean that RDRP of positive and NS RNA viruses have intrinsic different properties regarding their ability to use ribavirin as a substrate and template? In addition, the data shown in Fig 6 would suggest that at least for VSV, and perhaps the L polymerases from other NS RNA viruses, the antiviral activity of ribavirin is mediated by its ability to inhibit RNA elongation. This should be discussed in the context of a proposed mechanism of action for ribavirin based on its potential mutagenic activity.

Ribavirin is incorporated during copying of template by the poliovirus polymerase and templates containing ribavirin can be utilized by the poliovirus polymerase. Our data suggest that for VSV one of the mechanisms by which ribavirin inhibits the polymerase is through blocking elongation. Since we only observe this inhibition at high concentrations of RTP that would be difficult to achieve in a

physiologic context we are reluctant to draw conclusions about any potential mechanism of antiviral activity of ribavirin against VSV. Moreover, without comparing several positive and negative-strand RNA viruses we feel it premature to generalize about whether there are strand-specific differences in the ability of RTP to interfere with RNA synthesis by positive vs negative-strand RNA viruses. Nevertheless, we now specifically mention error catastrophe as a possible mechanism of action of ribavirin (see lines 410-423).

3) In the discussion section the authors proposed, based on their results, that N and P play regulatory roles to ensure authentic copying. However, the present work did not include per se experiments that would allow the authors to assess the role of N. In this regard, it would have been interesting to incorporate experiments where the authors examined, in addition to P, the effect of N on initiation and processivity of RNA synthesis by the VSV L polymerase.

As requested by the reviewer we have now evaluated the role of N in polymerase processivity by comparing the activity of the P-L polymerase complex on an N-RNA template to its activity on naked RNA. To do this, we elected to use a 50-nt naked RNA. This is because a well-characterized transcript made by the polymerase complex is the 47-nt leader RNA. As expected we see production of this leader RNA from the N-RNA template, with some evidence for premature termination of RNA synthesis (Figure 5D). In marked contrast, we see abundant termination within the first 21-nt for the P-L complex on the naked 50-nt template, with little evidence for synthesis of the leader RNA (Figure 5D). Thus the presence of N-protein on the template dramatically alters the properties of the P-L polymerase complex. This is described in lines 219-228.

4) In some instances in the discussion section the authors have generalized their conclusions, based on their findings with VSV L, to the L proteins in general of all NNS RNA viruses, whereas there is some evidence suggesting that L polymerases from different NNS RNA viruses may have some differences in terms of their template requirements. Likewise the discussion about the mechanisms by which L may displace N from the template, required for L-mediated RNA synthesis, assumes that L would copy the RNA template as a monomer, but there is evidence suggesting that for at least for some NNS RNA viruses the functional polymerase requires some degree of L oligomerization, which should be further discussed.

We agree with the reviewer that there may be differences in the requirements for some NNS RNA viruses. However, as we point out in the introduction, it has been very difficult to perform experiments to probe the mechanisms of initiation in these viruses. VSV is currently the only member of the order for which robust RNA synthesis can be reconstituted in vitro. We also devote an entire paragraph of the discussion (lines 370-384) describing experiments that propose an altered mechanism of initiation for respiratory syncytial virus.

There is no direct evidence, to our knowledge, that the functional polymerase of any NNS RNA virus requires oligomerization. There is evidence that mutations in different regions of the polymerase can complement one another (it is not clear how), and some co-immuneprecipitation experiments support the possibility of an oligomer. Our own previous biochemical and electron microscopic characterization of the VSV polymerase complex shows that the polymerase predominantly is present as a monomeric L-P complex, with some L-P dimers that likely are a reflection of the oligomerization status of P. Within those dimers the arrangement of the two separate L molecules is variable with respect to one another, and it is not clear whether they would become ordered on template binding. Since the available evidence indicates that the polymerase active site resides within a single L protein molecule, we have restricted our comments regarding N protein displacement to such a polymerase complex.

5) I think that the discussion section could be shortened without impacting its scientific content, as it contains some redundant information already presented in the results section of the paper.

In revising the text we attempted to eliminate redundancy while including new discussion as requested by the reviewer in points 1-4 above.

Referee #2:

This contribution to our understanding of initiation of synthesis by the VSV L polymerase breaks new ground, as this is the first report claiming that some synthesis, albeit modest, can take place with an RNA template that is not encapsidated by the viral nucleoprotein. Morin et al., show that L protein only synthesizes short products from a 19 nt long template RNA that encodes the 3' end of the viral genome. This is clearly not what the polymerase does in the natural context of VSV infection but the findings do inform our understanding of the catalytic mechanism and what L can do on its own. The paper is well written and the data are well presented. The authors' main conclusion however, i.e., that L can in fact initiate templated synthesis from the 3' end residue of the template, lacks firm support. This deficiency might well be resolved by providing sequence data of the main products, and establishing whether L can synthesize the first phosphodiester bond (AC dinucleotide) independently of template. The latter could explain why recent findings using a related virus (RSV) strongly suggest that L initiates synthesis in a non-templated fashion. The authors should address the following points.

We thank the reviewer for their comments that this work breaks new ground.

1.Fig. 1 - The gamma A-labeled products were synthesized under limiting ATP conditions which could explain why the band pattern is very different from the alpha-G products even when accounting for the two G residues. But the band pattern of alpha-G products at 1.5 mM ATP differs substantially between Fig. 1A, Fig. 1B and Fig. 2B. Even putting aside this variation, the authors should determine the actual sequence of the major product bands before concluding that initiation must occur at the very 3' end of the template, especially since early studies using the encapsidated template showed strong evidence of internal initiations *in vitro*.

The template used in Figure 2B contains the substitution U1C, which will also contribute to mobility differences, and now places a high [GTP] required for efficient initiation.

The conclusion that initiation starts predominantly at position 1 is supported by several lines of evidence:

- *(i) As requested by the reviewer, we verified that the sequence of the transcripts made by the polymerase correspond to initiation at the 3' end of the genome. In a new figure 1B, we cleave the products of synthesis with RNase A and show that they generate a labeled 2-nt, 6-nt and 4-nt products which would be expected for initiation at the 3' end of the wild type template. (see also lines 119-132)*
- *(ii) Figure 2A shows that the polymerase can only initiate RNA synthesis with a purine (ATP/GTP) with a preference for ATP. It is noteworthy that if indeed, internal initiation occurred on the template at any significant level, we would observe products in Figure 2B with gamma labeled ATP or in Figure 4B with gamma labeled GTP. Since such products are not observed, any internal initiation must be highly inefficient, which contrasts with that observed at the authentic UGC present at the 3' end of the genome.*
- *(iii) When we displace the authentic 3' terminal sequence from the end of the RNA, we observe some RNA synthesis – suggesting internal initiation can occur - but the levels are significantly reduced (Figures 3 and 4).*

2.Fig. 1 - It is curious that the observed synthesis required high concentrations of both ATP and CTP while earlier studies using encapsidated templates clearly established that this requirement pertained to ATP only. Do the authors have an explanation for this?

In earlier work, Testa and Banerjee demonstrated that only ATP is required at high concentration for transcription by L, and show that ATP is essential during initiation of RNA synthesis. Nevertheless, in this work they examined only the initiation requirement for ATP and not the other NTPs. Thus, our results show the requirements by L for the early step of initiation, and L can have different specific requirements during the different steps of the transcription process.

3.The gel analyses shown in the various figures do not identify dinucleotides. Given the recent findings showing that the RSV polymerase can correctly initiate replicative synthesis *in vivo* on genome templates lacking the 3' end residue, or on antigenome templates lacking the first two

residues, the authors' should exclude the possibility that L protein only can catalyze synthesis of the AC dinucleotide (and possibly other dinucleotides) in the absence of template ("preloaded" L). If this were the case, the authors' conclusions would need revision.

As requested by the reviewer, we examined whether the polymerase can catalyze the synthesis of a dinucleotide in vitro in the absence of template. We do not observe evidence for the synthesis of a pppAC dinucleotide in the absence of template (Figure 1B).

4.Figs. 2 and 3 - The inhibitory effects of base substitutions at the first two positions of the template (Fig. 2), as well as the observed synthesis when appending UG or UGC at the 3' end of a random template, are consistent with L initiating at the 3' end and the high concentration requirements for the complementary nucleotides but do not exclude a "preloaded" L initiating templated synthesis subsequent to catalyzing dinucleotide formation.

As pointed out in response to points 1 and 3 above, we have no evidence for the production of transcripts that are not templated as determined by their sequence, nor do we have evidence for the synthesis of a pppAC dinucleotide (Figure 1B).

5.Fig. 4 - The results of adding an extra one, two, or three nucleotides at the 3' end of the template are difficult to interpret. The authors claim this reflects internal initiation at the proper U residue corresponding the virus genome 3' end, but this also emphasizes that L can easily start internally and, absent sequence information of the product bands, where initiation occurs remains unclear.

The mobilities of the products of RNA synthesis are unaltered following addition of 1-3 nt at the 3' end of the RNA indicating that they are the same sequence (if initiation occurred elsewhere the products would have a different mobility). Moreover, we show (see point 1) that the initiation from the 19-nt template is templated and occurs opposite nt-1.

Referee #3:

In their paper, Morin et al. have characterized the mechanism of RNA synthesis initiation by the vesicular stomatitis virus (VSV) polymerase (L). For this, they have taken advantage of their ability to purify a His-tagged recombinant L protein expressed in SF21 cells to establish a very simple *in vitro* system. In this system, they only incubate naked RNA with L polymerase. This differs from previous systems to study RNA synthesis *in vitro* for nonsegmented negative-strand RNA viruses, which are limited by the necessity to purify the N-RNA template from viral particles or from cells. Particularly, with these systems, the influence of the template sequence cannot be analyzed.
Here, they demonstrate that vesicular stomatitis virus L initiates synthesis via a *de novo* mechanism that is independent of N or P and needs a high concentration of the first two nucleotides and specific template requirements. They also show that presence of P enhances processivity. Finally, they use their system to characterize the mechanism of action of ribavirin demonstrating that their experimental system represents a powerful method for understanding how inhibitors of RNA synthesis function.
In general, the paper is well written, easy to read and the experimental data are convincing. Nevertheless, I have some remarks that have to be addressed by the authors:

We thank the reviewer for their appreciation that this is a powerful method for understanding how inhibitors of RNA synthesis function.

Major points:

1) In figures 1A and 4B, the migration profiles are very different when the neo-synthesized RNA is labeled with [32P] ATP or with [32P] GTP. They should look the same (as it is the case in figure 2B when the neo-synthesized RNA are labeled with [32P] GTP or with [32P] GTP).

See response to the same point raised by reviewer 2.

2) As my previous remark raises some question about the reproducibility of the assay, I would like to see the error bars on figures 1C, 2C and 6C.

As requested, we now include error bars.

3) The authors indicate that the reactions mixtures were incubated at 30ºC for 3 hours in presence of L. I would like to see some kinetic datas that justify this reaction time (and may be a comparison of these kinetics with those obtained in the previous experimental systems using an N-RNA template)

We now include a time course of the products of RNA synthesis (Supplemental Figure 1) and we compared the products of synthesis from an encapsidated template (Figure 5D). The inclusion of the encapsidated template for comparison further underscores that the template associated N protein is an important processivity determinant.

Minor points:
1) In the discussion (p14, line 257), the authors have written "Since the L-P complex itself is not fully processive on naked RNA, our findings suggest that the template associated N may also represent an important processivity factor for the RdRP". In absence of any experiment performed with N (i.e. using an N-RNA template), I would avoid to write this sentence. Processivity might be as well influenced by local nucleotide concentration and by any cellular co-factors (and even by the phosphorylation state of P).

We have now shown that the template associated N protein facilitates processivity, and we have eliminated the requirement for P protein phosporylation in our in vitro RNA synthesis assays. See responses to reviewer 1.

3rd Editorial Decision 01 December 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by two referees whose comments are enclosed. As you will see, all referees express interest in your manuscript and are in favor of publication, pending satisfactory minor revision.

I particularly would like you to comment on the point raised by referee #2 in a point by point letter. I leave it up to you whether to modify the main manuscript file about this or not.

We generally allow 2 weeks for minor revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #2:

The authors should be commended for addressing all of the reviewers' comments, which significantly strengthen the conclusions of their study. The legend to figure 5, part D states that L alone was used with the various templates although the L/P complex was undoubtedly used as stated in the text. This minor correction however does raise the possibility that L alone can in fact synthesize some products with the N-RNA template. Have the authors tested this?

Referee #3:

In their revised version of their paper, Morin and colleagues have taken into account my remarks (and those of the other referees).

1) Using RNase A, they have generated labeled 2-nt, 6-nt and 4-nt products as expected if initiation occurs at the 3' end of the wild type template.

2) They have included controls with a P-mutant known to lack the ability to associate with the L protein (P 106).

3) They have also included an experiment showing that N is indeed a major factor for L processivity.

4) They have now added error bars on their graphs where needed.

From my point of view, the paper is now suitable for publication.

2nd Revision - Authors' Response 01 December 2011

We are delighted that the referees agree we have fully addressed their comments. To address the minor points raised by referee 2.

(i) The legend to figure 5, part D states that L alone was used with the various templates although the L/P complex was undoubtedly used as stated in the text.

We have now corrected the legend to Figure 5 to show that indeed it was the L-P complex that was used on the N-RNA.

(ii) This minor correction however does raise the possibility that L alone can in fact synthesize some products with the N-RNA template. Have the authors tested this?

Emerson & Wagner, 1973 (Journal of Virology 12:1325-1335) showed that L alone could synthesize RNA when added back to purified N-RNA. Subsequent work by Emerson & Yu, 1975 (Journal of Virology 15:1348-56) showed that both P (formerly known as NS) and L were required to reconstitute RNA synthesis on the N-RNA template. The explanation for this is that P does double duty as both an essential component of the polymerase complex (L-P) and is also associated with the N-RNA. It is very difficult to remove all traces of P from the purified N-RNA template (Emerson and Yu, 1975), and small quantities of P are sufficient to permit RNA synthesis. We too have observed that L alone can synthesize a low level of RNA when incubated with the N-RNA template, which we attribute to traces of residual P.