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RNA polymerase I catalysed transcription of insert viral cDNA

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ABSTRACT

RNA polymerase I has been used for transcription of influenza hemagglutinin (HA) cDNA precisely linked in the anti-sense configuration to both mouse rDNA promoter and terminator segments. In transcription reactions based on Ehrlich ascites cell nuclear extracts, specific uniform RNA products are synthesized in high rates that are comparable to original rDNA template transcriptions. Primer extension reactions show the 5′ ends of these RNA transcripts to be located exactly at position +1, corresponding to the 5′ end of negative strand HA viral RNA. RNA 3′ ends in a first series of constructs were found extended beyond the accepted location of pre-rRNA 3′ ends, in using both hybrid cDNA and original rDNA templates. But upon deletion of six basepairs from the rDNA termination region RNA polymerase I transcription has been adapted to yield correctly terminated influenza viral RNA *in vitro*. This result has been confirmed in an *in vivo* experiment via synthesis of an anti-sense viral RNA molecule containing the chloramphenicol acetyltransferase (CAT) gene, which in turn is recognized at its terminal sequence by viral RNA dependent RNA polymerase for plus strand mRNA synthesis and expression of CAT activity.

INTRODUCTION

Transcription of ribosomal RNA (rRNA) genes is catalysed by RNA polymerase I (1). Corresponding promoter sequence elements have been demonstrated immediately upstream of the initiation point for 45S precursor rRNA (2–4), but their exact boundaries are not yet precisely defined. One controversial point concerns the possibility of regulatory elements overlapping distal coding sequences, up to approximately position +10 (5,6). Among the upstream control elements, a specific sequence around position −165 including several transcription factor binding sites, has been studied in detail in the mouse system (7–10). Surprisingly, this sequence element bears some resemblance to distal terminator elements and contains a *SalI* restriction site, too. Accordingly it has been termed T_0 (11).

While initiation of RNA polymerase I transcription appears to be roughly similar in its requirements to RNA polymerase II initiation, the molecular events resulting in specific termination of pre-rRNA transcripts are clearly different. The entire pre-rRNA sequence extends beyong the coding sequence of the mature 28S rRNA by 565 nucleotides (in the mouse system; 12). This point is 22 bp ahead of the first 18 bp Sal box element which belongs to a conserved cluster of eight such sequence repetitions within positions +587 to +1178, again in relation to the 3′ terminal 28S coding position in the mouse system. These elements have been found to be essential for termination and to serve as binding sites for termination factor TTF-I (13), as a tight series they may also constitute a replication barrier protecting rDNA transcription against oppositely moving replication forks (14). It was also shown by nuclease S1 analysis that the 3′ end of rRNA precursor molecules appears to be the product of a two-step process: a primary termination reaction only 12 nucleotides upstream of the first Sal box (final 11 bp remaining untranscribed) is followed by a specific trimming reaction during which 10 nucleotides are removed, and a secondary 3′ end of the pre-rRNA molecules is resulting in that more upstream position (15).

RNA polymerase I transcription has not so far been used in hybrid constructions for expression of foreign RNA molecules. Since the 5′ cap-structure and 3′ poly-A tail are missing from the resulting transcription products such RNA molecules could not function as messenger RNAs, but because of their structure might be adapted to serve as viral RNA molecules if expressed from corresponding cDNA fragments inserted between rDNA promoter and terminator segments. Since at least most of the promoter and terminator elements appear to be located in external positions relative to the insert, this may be a feasible approach to the expression of viral RNA molecules and would have the advantage of the high rate of transcription expected for an rRNA-derived system. As an example for a negative strand vRNA expression by RNA polymerase I transcription influenza viral HA cDNA was chosen as an insert.

MATERIALS AND METHODS

Plasmid constructions

In addition to standard vectors, several derivatives of a basic construct, pHK236 (M.Kröger, unpublished), have been used as plasmid vector DNAs for controlled, IPTG dependent expression. In pHK236, which contains the *lacI*^{q}-gene, a synthetic p_{tac}

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promoter is followed by a standard multilinker, and two terminators derived from the *rrn*B gene of *E.coli* (see pJF118, (16)) complete the expression cassette within a pAT153 type plasmid.

Plasmid template constructions included mouse rDNA promoter and terminator segments: positions −254 to −1, and +1 to +179 relative to precursor rRNA transcription start and proposed stop positions (or +566 to +745 relative to the 28S rRNA 3′ end in the case of the terminator segment), respectively. These segments were arranged to flank hybrid cDNA from human influenza RNA segment 4 (hemagglutinin) of the two influenza virus isolates A/Victoria/3/75 and A/Aichi/2/68 (H3N2; kindly provided by W. Fiers, Gent). Viral DNA inserts included the complete hemagglutinin (HA) segment of 1765 bp (pHL746) or internal deletions thereof: pHL757 to pHL759, see Fig. 1. In reconstructing the border regions by oligonucleotide synthesis slight variations were introduced at positions 24 and 30 (TTTAAATACTAGT instead of TTTAATTACTAAT), and 1749/1750 (AATATT instead of AATTAT) of the HA vDNA sequence for the insertion of restriction sites. Also, the sequence upstream of the first Sal box was slightly varied in pHL746 (but not in the pHL832 to pHL880 series) to take into account the consensus of all eight Sal box segments, see Fig. 6B.

In pHL926 the chloramphenicol acetyltransferase (CAT) gene was inserted as a coding substitute exactly between the translation initiation and stop codons of the hemagglutinin reading frame, in anti-sense direction relative to RNA polymerase I transcription. A mouse ribosomal DNA clone with extensive rDNA internal deletion (pPTBH: rDNA minigene) was obtained from I. Grummt (17).

In vitro transcription

Ehrlich ascites cells grown in the mouse peritoneal cavity were cultured in RPMI medium complemented with 5% of newborn calf serum. Cells were harvested at a density of $1 - 1.2 \times 10^6$/ml. S-100 extracts prepared according to Weil et al. (18), and nuclear extracts according to Dignam et al. (19) were standardized in their transcription activity using the pPTBH: rDNA minigene template. For *in vitro* transcription experiments, 30 ng of template DNA were incubated in a final volume of 50 µl in the presence of a mixture of nuclear and S-100 extracts. Reaction mixtures contained 12 mM Hepes (pH 7.8), 85 mM KCl, 0.12 mM EDTA, 5 mM $MgCl_2$, 10 mM phospho-creatine (Sigma), 0.66 mM each of ATP, CTP and GTP, 12.5 µM of UTP, and 3 µCi of [alpha-^{32}P]-UTP (3000 Ci/mMol). Reaction mixtures were incubated for 60 min at 32°C. Synthesis was terminated by addition of 200 mM sodium acetate (pH 5.5), 0.4% $NaDodSO_4$, and 50 µg of tRNA. RNAs were extracted with phenol and chloroform, precipitated with isopropanol, and fractionated on 6% denaturing acrylamide gels together with ^{32}P-labelled DNA size markers.

Primer extension analyses of RNA 5′ ends

Primer extension analyses of RNA molecules transcribed *in vitro*, and of viral RNA from strain A/Victoria/3/75 isolated from embryonated eggs were performed to determine the 5′ ends of various transcripts, with influenza vRNA serving as a control. To samples containing the RNA products 10 mM vanadyl ribonucleoside complex and 1 ng of 5′-^{32}P-labelled primer oligonucleotide (5′-pTGGGCCTGCCAGAGAGG-3′, specific activity 0.5×10^6 cpm/ng oligonucleotide) were added, the mixtures were phenol/chloroform extracted and isopropanol precipitated. Dried pellets of nucleic acids were dissolved in 25 µl of hybridization buffer (80% deionized formamide, 40 mM Pipes, pH 6.4, 0.4 M NaCl, 1 mM EDTA), denatured at 80°C for 5 min and incubated at 37°C for 3 hours. After hybridization the samples were precipitated again and dissolved in 25 µl reverse transcriptase reaction mix containing 80 mM Tris−HCl, pH 8.3, 10 mM $MgCl_2$, 80 mM KCl, 4 mM DTE, 0.5 mM each of dATP, dCTP, dGTP, TTP and 15 u of AMV reverse transcriptase (Boehringer, Mannheim). The primer extension reaction mixture was incubated at 37°C for 40 min, and the reaction was stopped by incubation at 65°C for 5 min and further treated with 1 µg RNAse A (DNAse free) at 37°C for 20 min. Newly synthesized cDNA was purified by phenol/chloroform extraction, precipitated with isopropanol, dissolved in 100 µl deionized water and again isopropanol precipitated.

The primer extension products were resolved by 6% polyacrylamide/8 M urea gel electrophoresis, with a standard Sanger sequencing ladder (20) of the corresponding M13 clone primed with the same 5′-labelled oligonucleotide as used in the reverse transcriptase reaction. The resulting ^{32}P-labelled products were visualized by autoradiography.

As a control a similar primer extension reaction was performed using influenza viral RNA (prepared as a mixture of eight RNA segments from influenza A/Victoria/3/75) isolated from embryonated eggs, again in comparison with a corresponding sequencing ladder.

Nuclease S1 mapping of RNA 3′ ends

In vitro synthesized transcription products have been hybridized against surplus amounts of a specific DNA fragment spanning the HA cDNA/rDNA 3′ boundary region, between *Spe*I and *Sal*I restriction sites (177 bp). Upon DNA denaturation at 80°C hybridization was carried out for 4 hours in 25 µl of hybridization buffer (80% formamide, 40 mM Pipes, pH 6.4, 0.4 M NaCl, 1 mM EDTA) at 30°C, i.e. 3°C above the melting point of the DNA fragment as determined under these conditions. For nuclease S1 reaction the hybridization mixture was diluted into cold 225 µl S1 buffer (30 mM Na-acetate, pH 4.5, 0.3 M NaCl, 3 mM $ZnCl_2$), and after addition of 384 u S1 nuclease (Pharmacia) incubated at 21°C for 60 minutes. After phenol extraction, precipitation of protected heteroduplex fragments and RNAse treatment an oligonucleotide was added for primer extension DNA synthesis by AMV reverse transcriptase (^{32}P-TGGCACCGCATGATGTC-3′, homologous to positions +1625 to +1642 of HA vRNA), and after denaturation the reaction was started in hybridization buffer at 30°C under conditions as described above. The complete procedure was also done in parallel for total influenza viral RNA (mixture of eight segments). For a Sanger sequencing ladder the same primer was used together with an M13mp18 template DNA covering the same region.

RNA product ligation by T4 RNA ligase

In vitro synthesized transcription products have been treated in 25 µl ligase buffer (50 mM Tris−HCl, pH 8.0, 10 mM $MgCl_2$, 10 µg/ml BSA, 25% polyethylene glykol PEG 8000) with 40 µM ATP and 5 u T4 RNA ligase at 25°C (21). After phenol extraction and precipitation first-strand DNA synthesis was initiated with 250 u of AMV reverse transcriptase (Boehringer) in the presence of 100 u RNAsin as described above, using oligonucleotide 5′-TGGGCCTGCCAGAGAGG-3′ complementary to positions +80/+63 in the HA transcripts for starting that reaction in a

volume of 20 μl (5 mM $MgCl_2$; 50 mM KCl; 10 mM Tris HCl, pH 8.3; 1 mM dATP, dCTP, dGTP, dTTP, each; 0.75 μM oligonucleotide). After inactivation of the enzyme at 99°C the reaction volume was increased to 100 μl and converted into PCR conditions (final concentrations: 2 mM $MgCl_2$; 50 mM KCl; 10 mM Tris HCl, pH 8.3; 0.2 mM dATP, dCTP, dGTP, dTTP, each; 0.15 μM oligonucleotides I and II, each; 2.5 u Taq polymerase per 100 μl), including addition of the upstream primer as described above, homologous to positions +1625 to +1642 of HA vRNA. After 35 cycles of amplification in a Perkin Elmer thermocycler DNA products have been isolated, cleaved with restriction endonucleases appropriately and cloned into pUC19. 32 clones have been isolated and sequenced across their 3′ end/5′ end fusion points.

Lipofektin DNA transfection and CAT assay

For transfection B82 cells (a mouse L cell line) were used. 10^7 cells were treated with 5 μg pHL926 DNA mixed with 60 μg Lipofektin (Lipofektin™ reagent, GIBCO/BRL) in serum-free medium. After 3 hr of incubation the medium/Lipofektin/DNA mixture has been removed, and further incubation was performed with DMEM medium for several hours. Prior to the start of transfection the cells have been infected with influenza virus in PBS (influenza A/Asia/57, grown in embryonated eggs) at an moi of 1 for 1 hr, followed by a recovery period of 60 minutes in DMEM medium.

CAT assays were performed as described by Gorman et al. (22). Assay mixtures contained: 0.1 μCi [^{14}C]-chloramphenicol, 20 μl 4 mM Acetyl-CoA, 25 μl 1 M Tris−HCl (pH 7.5) and 50 μl of cell lysate, total volume 150 μl; incubation was continued for 18 hr, before reaction products were separated by chromatography and autoradiographed.

RESULTS

Cell-free transcription of rDNA/HA cDNA template DNAs by RNA polymerase I

We have constructed a hybrid mouse RNA polymerase I transcription system for *in vitro* synthesis of influenza hemagglutinin (HA) vRNA. For that purpose the complete HA cDNA (1765 bp) was fused exactly between a mouse rDNA promoter sequence extending from −254 to −1 relative to the startpoint of the 45S pre-rRNA (12), and a hybrid mouse rDNA terminator sequence containing two rDNA terminator signals T_1 and T_2 with a stan-dard 55 bp interval (positions +566 to +745 relative to the 3′ end of 28S rRNA). DNA fragments extending across the two fusion points had to be reconstructed by oligonucleo-tide synthesis, and two point mutations each in the HA cDNA sequence approximately 25 bp from both ends (see Methods) allowed introduction of extra cleavage sites, including an *Spe*I sequence close to the AUG-initiator sequence, i.e. in anti-sense direction near the vRNA 3′ end. In addition to full

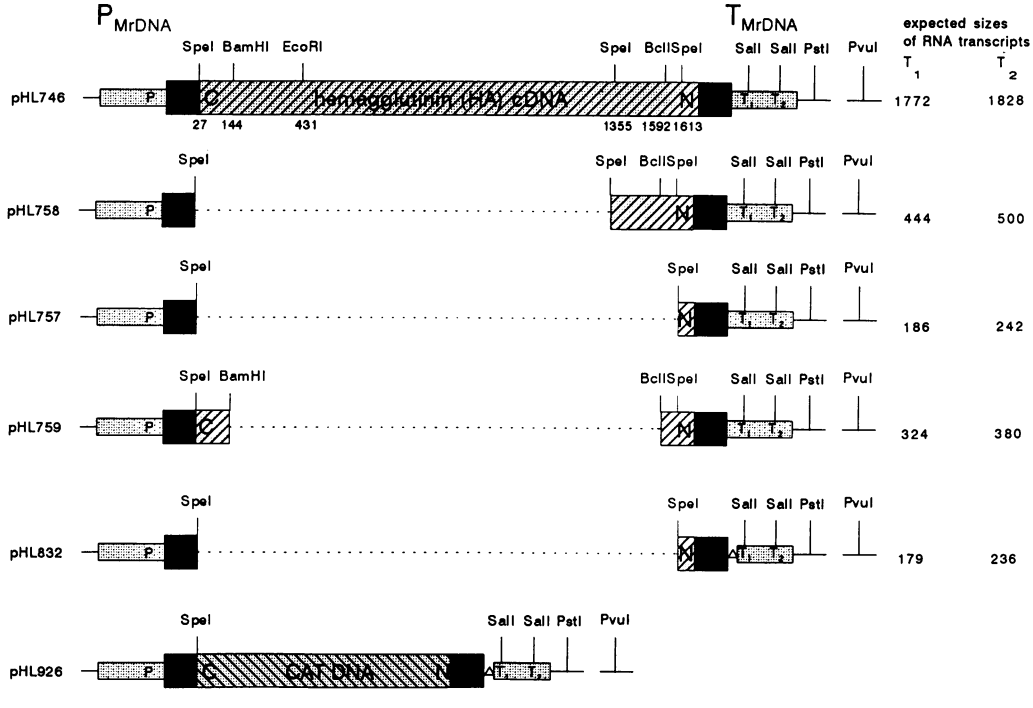

Figure 1. Plasmid map of hybrid RNA polymerase I transcription systems with HA cDNA derived inserts, in anti-sense orientation for expression of vRNA-like molecules. Full size HA cDNA (pHL746), or derivatives with internal deletions (pHL757, pHL758, pHL759), or with internal plus external deletions (pHL832). pHL926 carries a hybrid insert with the HA reading frame exactly exchanged for the chloramphenicol acetyltransferase (CAT) coding sequence, with both viral terminal cDNA sequences (in black) retained in place; again an external 6 bp deletion is marked by △. Wide boxes indicate either HA cDNA or CAT DNA with terminal uncoding (black) and anti-sense coding (hatched) segments, smaller boxes represent mouse ribosomal promoter or terminator segments, with P and T_1, T_2 designations for promoter regions and Sal boxes, respectively. Thin lines indicate flanking sequences of vector plasmid pHK236. Positions of restriction sites used for HA internal deletions are indicated below pHL746, additional sites refer to template cleavage points as used in run-off transcriptions or for other purposes. N and C indicate the locations of start and stop codons in anti-sense oriented reading frames. Boundary regions are not drawn to scale. Tabulated transcript sizes for T_1 and T_2 termination refer to experimental results in Figs. 2 and 6A. For sequence differences between pHL757 and pHL832 (pHL926) in the terminator region (marked by △) compare Fig. 6B.

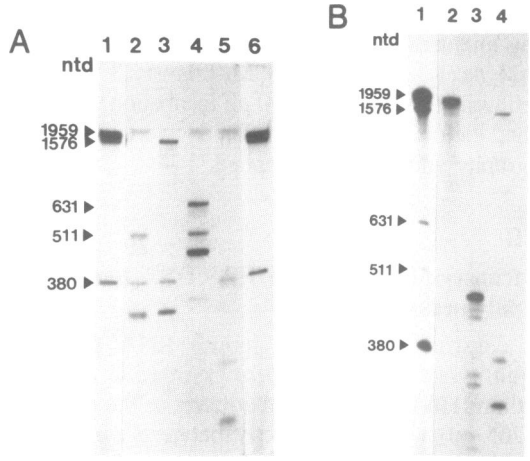

Figure 2. *In vitro* transcription of HA cDNA with RNA polymerase I activity present in nuclear extracts. (**A**) Autoradiogram of *in vitro* transcription products obtained from various HA internal deletion templates: Lane 2—pHL759/*Pst*I; lane 3—pHL759/*Pvu*I; lane 4—pHL758/*Pst*I; lane 5—pHL757/*Pst*I; lanes 1,6—^{32}P-labelled DNA size-marker. (**B**) Autoradiogram of RNA products resulting from full-length HA cDNA transcription: Lane 2—pHL746/*Pst*I (external run-off); lane 3—pHL746/*Eco*RI (internal run-off); lane 4—pHL759/*Pvu*I; lane 1—^{32}P-labelled DNA size-marker.

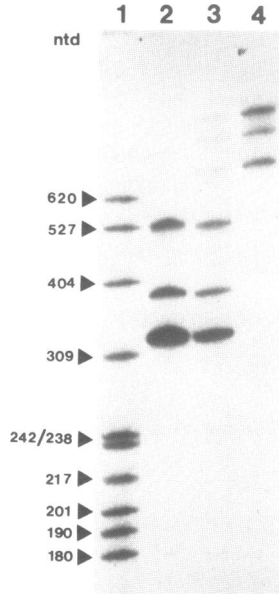

Figure 3. Competitive transcription experiment of hybrid HA cDNA template pHL759 versus original rDNA template pPTBH. Autoradiogram of transcription products. Lane 2—pHL759/*Pst*I; lane 3—pHL759/*Pst*I + pPTBH/*Eco*RI (1:1); lane 4—pPTBH/ *Eco*RI; lane 1—DNA size marker: ^{32}P-labelled pBR322/*Hpa*II.

size HA cDNA/rDNA plasmids (such as pHL746) shorter versions have been created with internal deletions of different lengths, see Fig. 1.

In a cell free transcription system based on nuclear extracts from Ehrlich ascites cells (1,12,19) each of these constructs was expected to generate two specific transcripts terminated at each of the two terminators 55 bp apart on the template DNA. As shown in Figures 2A and 2B three distinct transcription products are observed in each of these hybrid *in vitro* transcription systems. Since the template DNAs have been linearized at downstream restriction sites, a third signal will result from run-off transcription and will represent the fraction of transcription reactions which did not undergo termination. Viral RNAs or deletion derivatives thereof appear to be transcribed in high numbers and from a unique initiation site within the hybrid promoter. Also, termination reactions seem to occur in a precise and specific manner, since RNA transcripts are of sizes predicted by the lengths of the respective HA cDNA templates, and each pair displays a constant difference in length reflecting the distance of 55 nucleotides between the two terminator signals. Similarly, the sizes of the run-off transcripts are also in accordance with the restriction sites chosen for linearization of the template DNAs (pHL759/*Pst*I is expected to yield a product 518 nucleotides in length, pHL758/*Pst*I DNA: 638 nucleotides, pHL757/*Pst*I DNA: 380 nucleotides). While most of the *in vitro* transcription reactions have been done with shorter cDNA templates for better product analyses, full-size HA cDNA templates of 1765 basepairs similarly gave rise to three clear signals (Fig. 2 B).

To compare the efficiency of RNA polymerase I transcription of one of the hybrid templates (pHL759) versus an original rDNA minigene construct (pPTBH; see 17) roughly similar in size (transcripts of 324 versus 385 nucleotides) we used both templates together in competition assays. In several such transcription experiments equivalent amounts of template DNAs (30 ng in individual reactions and 2×15 ng in mixed reactions) or other ratios were mixed before adding S-100/nuclear extracts to start the reaction. The result of one of these experiments is presented in Figure 3. Both pPTBH linearized with *Eco*RI and pHL759 cleaved with *Pst*I resulted in a characteristic set of three product RNA bands. In the reaction shown the set of three pPTBH transcripts migrated aberrantly slowly (but distinctly separated from the pHL759 products) in gel electrophoresis, probably due to insufficient denaturation of RNA secondary structures.

Based on this and a series of other competition experiments it can be concluded that pHL759 DNA is able to compete with original rDNA minigene templates for factors needed for specific initiation and termination of transcription by RNA polymerase I.

Analysis of the vRNA transcript 5′ ends

To determine whether the mouse hybrid rDNA promoter is able to initiate transcription of HA viral RNA at the correct startpoint position +1, a primer extension analysis was performed using the mixture of *in vitro* RNA transcription products. This analysis yielded a single primer extension product for all of the *in vitro* transcribed RNA molecules of template pHL759 (see lane 1 in Fig. 4A). The unique transcription start site for all three transcription products, as determined from these primer extension analyses, is indicated by an arrow in Figures 4A and 4B. The position at which all transcripts initiate corresponds exactly to the +1 position as known for original rDNA templates, and is identical with the 5′ end of influenza viral RNA.

The 5′ terminus observed in a control primer extension reaction for authentic influenza HA vRNA yields the same position for the segment 4 (HA) 5′ end, see lane 2 in Fig. 4B. Both reactions are controlled using sequencing ladders obtained from corresponding M13mp18 clones, initiated with the same ^{32}P-end-labelled oligonucleotide as used in the primer extension reactions. The same unique initiation point has been confirmed in parallel reactions for two other of the hybrid HA cDNA templates including full-size pHL746 (not shown). Therefore,

Figure 4. Determination of the RNA product 5′ end from *in vitro* transcription of HA cDNA. (**A**) Autoradiogram of primer extension products: Lanes A,C,G,T—^{32}P-end-labelled Sanger sequencing ladder from a corresponding M13 clone; lane 1—primer extension product obtained from the RNA transcripts of template pHL759/*PstI*; lane 2—primer extension product of HA viral RNA obtained from influenza A/Victoria/3/75 RNA (mixture of eight segments). The last position of the primer extension product in lanes 1 and 2 is marked by an arrow on the right border. The corresponding thymidine (T) in the sequencing ladder is equivalent to nucleotide +1 in the coding strand of the HA cDNA construct and the 5′ terminal nucleotide (A) of HA vRNA. (**B**) Schematic representation of the primer extension reaction used: Lanes 1,2—DNA double strand representing the rDNA/HA cDNA hybrid initiation region; lane 3—5′ end of RNA transcript resulting from hybrid HA cDNA template/5′ end of HA viral RNA; lane 4—product of primer extension reactions, position of terminal nucleotide as determined in (A).

we conclude that foreign RNA molecules can be produced *in vitro* with exact 5′ termini in hybrid RNA polymerase I transcription.

Analysis of the vRNA transcript 3′ ends

For an exact determination of the 3′ ends of RNA polymerase I transcription products *in vitro*, nuclease S1 analyses have been performed. For that purpose an excess of a specific 177 bp DNA fragment extending across the expected T_1 3′ ends and up to the *SalI* restriction site within T1 (Fig.1) has been used for hybridization of *in vitro* synthesized vRNAs. Following treatment with nuclease S1 under mild conditions (e.g. for 60 min at 21°C; 1.5 u S1 per μl), S1-resistant heteroduplexes have been precipitated and after denaturation were used in a primer extension reaction along the complementary DNA strand. After incubation with nuclease S1 for degradation of single-stranded DNA tails the resulting 5′ end of hybridized DNA strands was expected to be equivalent to the 3′ end of the original vRNA transcript terminated at T_1. For comparison with the template sequence a sequence ladder initiated with the same ^{32}P-5′-labelled primer nucleotide was electrophoresed adjacent to the primer extension products. Also, a control experiment using original vRNA molecules of influenza virus (mixture of eight segments) has been done resulting in the expected 3′ position (not shown). Altogether two signals are expected in these analyses, see Fig. 5. One should originate from the mixture of T_1-terminated transcripts, and a second one can be predicted at the *SalI* cleavage site, since both T_2-terminated as well as run-off transcription products extend beyond the (first) *SalI* DNA recognition sequence; it serves as a kind of internal control. That latter signal is indeed observed at the cleavage position within the *SalI* sequence (and in small part immediately below) while the dominant lower signal representing the T_1-terminated vRNAs is observed at position +572, seven nucleotides beyond the expected 3′ terminal position at +565. This position is, therefore, located within the adjacent rDNA sequence, at the distal end of a U_6 sequence (and not proximal to it), 15 bp from the Sal box upstream boundary. Minor amounts of reaction products are observed for position +571, and with increased temperatures or S_1 enzyme concentrations, the primer extension

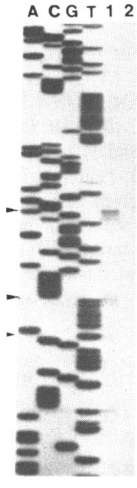

Figure 5. Nuclease S1 mapping of *in vitro* transcription product 3′ ends obtained from pHL759/*PstI* template DNA. Lane 1—primer extension products obtained after 5′-^{32}P-oligonucleotide initiated cDNA synthesis, in copying DNA template strands that remained protected during nuclease S1 degradation of single stranded tails, due to heteroduplex formation between transcript RNA/complementary strand DNA. Lane 2—control reaction using carrier tRNA only. Lanes A C G T—sequencing ladder of the respective region cloned into M13mp18, initiated with the same 5′-^{32}P-labelled primer. Arrowheads indicate (top to bottom): (1) *SalI* terminus of single strand DNA, protected by T_2-terminated or run-off transcripts; (2) dominant position observed for T_1-terminated transcripts; arrow indicates expected position for RNA 3′ end according to accepted interpretation of pre-rRNA termination point, in pHL757 equivalent to 3′ end of HA cDNA insert sequence.

signal becomes spread over several more proximal positions of that stretch of dA_6/rU_6 hybrid sequence (T_6 in the sequencing ladder of Fig. 5; not shown) indicating its instability against such S1 treatment under other conditions than those used in the definite experiments.

For confirmation of the result obtained for the 3′ ends of RNA products in RNA polymerase I transcription, we turned to another, independent technique. T4 RNA ligase treatment was used to convert RNA transcripts in part into circular monomer

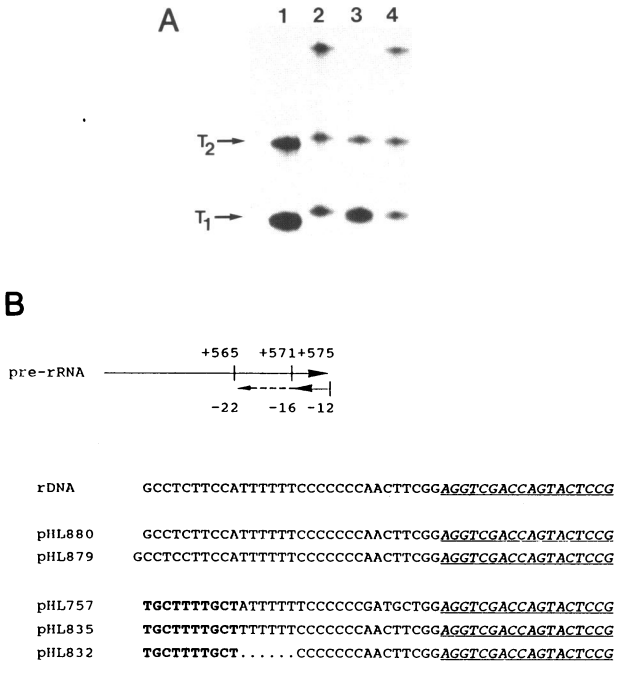

Figure 6. *In vitro* transcription of revised HA cDNA constructs with RNA polymerase I containing extracts. (**A**) Autoradiogram of *in vitro* transcription products obtained from 1) pHL832 DNA linearized by *NsiI* cleavage, 2) pHL879-*PstI*, 3) pHL835-*NsiI*, 4) pHL880-*PstI* separated in PAA gel electrophoresis. Positions of T_1 and T_2 terminated transcripts are indicated by arrows. (**B**) Comparison of the 3′ fusion regions of clones used in this study. Influenza cDNA segments are given in bold type, Sal box (TTF-I binding) sequences are underlined and italicized, dots indicate basepairs deleted. The scheme above represents the two step termination model for RNA polymerase I transcription, in its presently accepted (smaller arrowhead) and proposed (larger arrowhead) versions for the 3′ → 5′ processing reaction.

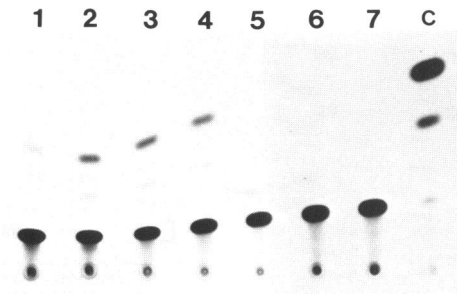

Figure 7. Expression of CAT activity in influenza virus infected and pHL926 DNA transfected murine B82 cells. 107ı cells each have been transfected by 5 μg pHL926 DNA in 60 μg Lipofektin from t_{-3h} to t_0, following infection with influenza strain: A/Asia/57 at moi: 1 from t_{-5h} to t_{-4h} in PBS, and a recovery period in DMEM medium from t_{-4h} to t_{-3h}. Cellular extracts have been prepared for CAT assay at (1) t_{3h}, (2) $t_{5,5h}$, (3) t_{8h} and (4) t_{11h} after Lipofektin treatment. Controls include (5) cells infected by influenza, but not transfected, (7) DNA transfected but mock infected, and (6) without either treatment, in every case isolated at t_{11h}. The control lane (c) marks the position of chloramphenicol acetyltransferase reaction products.

or linear dimer products via ligation of their 5′ and 3′ ends. Following first strand DNA synthesis by AMV reverse transcriptase, PCR amplification with a pair of primers oriented across the ligation bridge (i.e. towards both ends of the primary transcript) was used for getting increased amounts of the specific RNA bridge sequences that could be cloned and sequenced. In a series of 32 individual clones isolated and sequenced the 5′ terminal nucleotide as determined above was observed ligated to either T_1 or T_2-terminal sequences. In both groups a clear majority of 3′ terminal sequences (12 out of 19, and 13 out of 13) extended beyond the expected 3′ ends and well into the consecutive series of six or twelve uridine residues, respectively, but never across into further distal regions. Even though this RNA ligation technique due to RNAse trace activities remaining in nuclear extracts did not yield unequivocal results with regard to the exact location of the 3′ ends it is certainly supporting the result of the nuclease S1 determination, this time also including termination at T_2, in an undisturbed pre-rRNA termination reaction. In both cases a likely interpretation of the results presented is the lo-cation of the RNA 3′ end at the most distal residue in both series of uridines, i.e. in pHL757 DNA seven nucleotides beyond the expected T_1 pre-rRNA 3′ position according to published data.

Because of these results in a second series of hybrid viral rDNA clones the 3′ fusion sequence between HA cDNA and the Sal box upstream region has been changed accordingly, both into authentic T_1 rDNA (pHL880) or unchanged HA cDNA/rDNA fusion (pHL835), and by deletion of the dT_6 stretch coding for the 3′ rU_6 extension (see pHL832 in Figs. 1 and 6B). In *in vitro* transcription with RNA polymerase I containing nuclear extracts pHL832 transcripts proved to be terminated at unique positions for T_1 and T_2, approximately 6 nucleotides shorter than transcripts obtained from undeleted pHL835 or pHL880 DNAs (Fig. 6A). In this separation of RNA molecules pHL879 transcripts (an aberrant 1 bp insertion clone observed in oligonucleotide based constructions) served as an internal standard to prove that differences even as small as one nucleotide can be detected, and as a measure for the 185/179 and 242/236 nucleotide bias in transcripts expected for pHL835 and pHL832 T_1 and T_2 termination, respectively. The termination reaction appears not to be severely influenced in its efficieny or precision by deletion of six basepairs in pHL832 or other variations in that Sal box upstream region, including the deviations between pHL835 and pHL757, or between authentic rDNA templates and hybrid DNA sequences. The position of the RNA 3′ end for T_1-terminated pHL832 transcripts has been determined using the nuclease S1 method and has been found to be located at position 179, i.e. exactly at the 3′ end of this shorter version of the inserted HA cDNA sequence (not shown).

In vivo transcription of pseudo-viral CAT RNA

For an *in vivo* confirmation of the results achieved with transcription of viral cDNA molecules *in vitro* we constructed a reporter gene version of hybrid HA cDNA by precisely exchanging the HA coding region for that of chloramphenicol acetyltransferase, i.e. retaining the untranslated HA viral cDNA terminal regions (unchanged from pHL832) between the CAT gene sequence and DNA promoter and terminator elements, respectively (Fig. 1). In its general design, therefore, the CAT reading frame in pHL926 is inserted in anti-sense orientation with regard to the direction of transcription and is expected to give rise to an anti-sense CAT RNA upon transfection into murine cells and transcription by RNA polymerase I.

RNA molecules of this character cannot give rise to CAT activity, but upon superinfection by influenza virus are expected to yield plus strand mRNA molecules according to the cap-

Table 1. Comparison of 5'terminal RNA sequences transcribed by RNA polymerase I

	position									relative promoter activity (% of mouse wild type)	reference
	+1	+2	+3	+4	+5	+6	+7	+8	+9		
mouse, wild type	A	C	U	G	A	C	A	C	G	100	Grummt et al., 1982
rat, wild type	G	C	U	G	A	C	A	C	G	50	"
mouse, derivative	A	C	C	C	A	C	A	C	G	100	Clos et al. 1986
mouse, derivative	A	C	U	A	A	C	A	C	G	>100	Kishimoto et al. 1985
mouse, derivative	A	C	U	G	A	C	A	U	G	100	Kishimoto et al. 1985
mouse, derivative	A	C	U	G	A	C	A	C	A	100	Kishimoto et al. 1985
HA sequence	A	G	U	A	G	A	A	A	C	100	this paper

snatching mechanism of viral RNA polymerase transcription (23). This result is specific for influenza RNA type molecules and depends on viral polymerase recognition of terminal 'promoter' sequences of such RNA species. Upon transfection by pHL926 DNA and infection by influenza virus of murine B82 cells CAT activity has indeed been observed in cellular extracts, dependent on both steps of minus strand and plus strand RNA synthesis, and increasing during viral infection periods (Fig. 7). These results suggest that viral RNA molecules can indeed be transcribed from hybrid cDNAs by RNA polymerase I with perfect ends, *in vivo* as well as *in vitro*, and be recognized by viral RNA polymerase for specific plus strand RNA synthesis.

DISCUSSION

Synthesis of hybrid ribosomal precursor RNAs provides an attractive system for the transcription of foreign RNA molecules that are designed not to undergo translation. In particular for minus strand viral RNA molecules RNA polymerase I transcription has the advantage of resulting in RNA species without cap structures and poly-A tails, having instead 5' triphosphate and 3' OH ends of specific, template controlled sequence. The potential of this system could only be realized, however, if both terminal sequences of the inserted cDNA could be chosen freely, i.e. without structural limitations resulting from initiation or termination factor binding sites overlapping with either end of the rDNA coding sequences or restrictions by other steps of the mechanism.

In this report we have used a cell-free hybrid transcription system which consists of murine RNA polymerase I promoter and terminator sequences flanking an inserted full size or partially deleted cDNA of the influenza hemagglutinin gene. The murine rDNA sequences used contain the complete information for a selective transcription of mouse ribosomal precursor RNA as shown with similar internally deleted rDNA templates as controls. The rDNA/HA cDNA hybrid system served as a template for RNA polymerase I in the presence of S-100 and nuclear extracts prepared from rapidly growing Ehrlich ascites cells. The viral RNA transcripts obtained clearly showed that RNA polymerase I in the presence of cellular transcription factors efficiently recognizes the hybrid initiation site and selectively transcribes downstream cDNA sequences of different lengths.

By primer extension analysis it is demonstrated that the RNA polymerase I promoter fused to an HA cDNA sequence initiated transcription at the regular transcription start site (+1) known for rDNA. Due to the template fusion point chosen this position is identical with the first position of viral cDNA. All transcription products, therefore, begin with the correct 5' end of viral RNA molecules, independent of downstream template length. This should in principle be true also for inserted templates other than influenza cDNA.

Competition assays of rDNA between hybrid constructs and authentic rDNAs further indicate that both templates are transcribed in a competitive way, i.e. their transcription draws from the same initiation and termination factors in the extracts used. RNA polymerase I transcription of inserted foreign cDNA templates, therefore, appears to occur following the same initiation mechanism with the same accuracy of initiation, and also in similar yields as observed for original rDNA templates.

Although it has been claimed that sequences downstream of the initiation start site of RNA polymerase I are required for initation of transcription: +1 up to +9 (5), +1 up to +23 (24), or at least stimulate transcription: +1 to +11 (6) our results show that foreign cDNAs with entirely different nucleotide sequences can indeed be transcribed in the correct way. Because our results have been obtained so far with a single, foreign viral cDNA sequence, influenza HA, some minor downstream sequence requirements for RNA polymerase I cannot be rigorously excluded. Sequence similarities between rDNA and the viral cDNA sequence used occur at positions +1, +3, and +7 (Table 1). The presence of an adenosine (or purine) in position +1 appears to be a true requirement, but may be quite common for most template constructs being considered and should not cause severe limitations. Conservation of sequence at the two other positions may just be accidental and not in fact be required, since at least one rDNA sequence variant is known, which in position +3 contains a C instead of the common T residue without reduction in transcription rates (6).

With final proof awaiting a systematic variation of 5' terminal sequence positions we would like to suggest that downstream cDNA sequence restrictions beyond position +1 (purine) are unlikely in the light of these results. Therefore the polymerase I reaction might be useful for transcription of foreign DNA or cDNA templates without any serious sequence limitations at its 5' terminal region.

According to the *in vitro* transcription patterns with sharp bands for every individual transcript, similarly precise 3' ends of RNA molecules result from RNA polymerase I termination. Both methods used for determining that position: nuclease S1 treatment and primer extension, or RNA ligation and outside-in PCR amplification indicate the 3' ends of T_1 terminated RNA molecules for pHL757 to be located seven nucleotides more distal than expected. In a further analysis of *in vitro* transcription product sizes using molecules terminated within authentic rDNA sequence regions (pHL880, pHL879, see Fig.6) these transcripts are similarly observed to extend six nucleotides further downstream than presently accepted. In the RNA ligation method equivalent results have been obtained for pHL757, in this case

also including termination within the T_2 DNA sequence, which in hybrid constructs is unlikely to be disturbed in any way since the hybrid cDNA sequence is located much further upstream. Together with our own data in carrying out the S1 reaction at slightly elevated temperatures we would argue that previous results based on S1 nuclease treatment at temperatures above 21°C may have incorrectly assigned the terminus of pre-rRNA due to uncontrolled S1 degradation extending across that terminal dA_6/rU_6 heteroduplex sequence. This revised assignment of the pre-rRNA 3′ end has been further confirmed by deletion of that 6 bp template region in pHL832, which as expected yields transcription products six nucleotides shorter and located exactly at the viral cDNA 3′ end. The same is true for several other variations of that revised hybrid cDNA construct (G. Neumann and G. Hobom, unpublished data).

Expression of anti-sense viral RNA molecules by RNA polymerase I *in vivo*, and their recognition by influenza RNA polymerase for synthesis of plus strand messenger RNA molecules, which only *then* gives rise to CAT enzyme activity results in further proof for an (at least partially) correct formation of 5′ and 3′ RNA ends in that transcription. Recognition of terminal 'promoter' sequences by viral RNA polymerase is likely to be very sensitive for nucleotide additions and omissions, or deviations within that sequence (25,26), e.g. mRNA molecules of influenza are not recognized for viral RNA amplification, because of their terminal alterations due to cap-snatching and poly-A addition. Different from another technique based on *in vitro* transcription of influenza RNA (27) the method developed will allow *in vivo* expression of influenza viral RNA molecules carrying site-directed sequence variations.

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