ADENOVIRUS GENE EXPRESSION – A MODEL FOR MAMMALIAN CELLS

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Animal DNA viruses provide a powerful system for analysis of transcription and translation in mammalian cells. During productive infection, the adenovirus DNA enters the nucleus rapidly [1] and is transcribed into large RNA molecules [2–4]. Both early and late in productive infection nuclear RNA becomes polyadenylated [5] and RNA molecules which are destined to become messenger RNA (mRNA) are presumably cleaved before entering the cytoplasm [3, 6–9]. A switch from early to late gene expression occurs at the onset of viral DNA repliation and leads to quantitative as well as qualitative changes in the synthesis of viral mRNA. Late after infection the cytoplasm contains about 10-times more viral RNA than at early times [2,9] and early and late mRNA contain RNA species of different sizes as revealed by polyacrylamide gel electrophoresis [6–8]. In addition to the two classes of viral mRNA which are detected early and late after infection, a prominent low molecular weight species of virus coded RNA has been identified [10]. This RNA sediments at 5.5 S, contains 156 nucleotides and its sequence has been determined [11,12]. The 5.5 S RNA, whose function is unknown, is synthesized in large quantities, preferentially late during the infectious cycle. It has been shown [13,14] that 5.5 S RNA can be synthesized in vitro in isolated nuclei with a guanosine-tetraphosphate at its 5$^-$end. It is transcribed in the presence of low concentrations of α-amanitin, a drug which inhibits the synthesis of precursors to adenovirus mRNA [15]. The 5.5 S RNA therefore appears to be transcribed by RNA polymerase III [13,14] whereas the mRNA precursors probably are transcribed by an enzyme which is related to mammalian RNA polymerase II [15].

The synthesis of host cell RNA is unaffected at early times but is suppressed late in productive infection. Only 10–20% of the normal amount of ribosomal RNA is transported to the cytoplasm [9,16] late, although significant amounts of 45 S ribosomal precursor RNA appear to be synthesized [16]. Late after infection the synthesis of host-cell heterogeneous nuclear RNA (HnRNA) is suppressed [17]. Nearly all mRNA transported to the cytoplasm late after infection is of viral origin.

The sequences of mRNA which are synthesized early after infection persist in the cytoplasm late after infection although all early mRNA sequences are not synthesized late after infection [2, 18–22]. Rat cells transformed by adenovirus type 2 (ad2) contain viral mRNA sequences which are a subset of mRNA sequences synthesized early during productive infection. In some transformed cell-lines these sequences are derived from both complementary strands [19,23,24].

Digestion of viral DNA with bacterial restriction endonucleases, methods for complementary strand separation of the cleavage fragments and liquid phase RNA–DNA hybridization analysis have provided powerful tools for mapping of different classes of viral RNA on the adenovirus genome [17–19, 23–26, 27–28, 30].

It is also possible to select specific mRNA on restriction enzyme fragments and the genes for several early and late polypeptides have been localized on the ad2 genome by in vitro translation [31,32]. It is therefore now possible to analyze the expression of the adenovirus DNA genome in detail by a comparison of the maps for nuclear RNA, classes of messenger

RNA and the gene products. The present report summarizes our current results on adenovirus gene expression during productive infection.

Methodology

This will only be described briefly since details have been published in recent communications.

Our methods for preparation of adenovirus DNA and RNA have been described in detail elsewhere [8,17,18,22,25]. Techniques for separation of the complementary strands of ad2 DNA with poly(UG), for restriction enzyme cleavage, for strand separation of individual fragments and for hydroxylapatite chromatography have all been published in recent papers [17,18,22,25,27,29]. The l- and h-strand of ad2 DNA refers to the strand with light and heavy buoyant density in CsCl when complexed with poly(UG). Procedures for purification of cytoplasmic RNA by oligo(dT)–cellulose chromatography, labeling of viral RNA in vivo with [^{3}H]uridine as well as labeling of 5.5 S RNA in vitro with [^{125}I]iodine have been presented in other communications [18,22,25,29,30]. Methods for synthesis of RNA in vitro in isolated nuclei and polyacrylamide electrophoresis of low molecular weight RNA species have also been described [33]. In vitro protein synthesis was carried out in wheat-embryo extracts [34] and virus specific mRNA was selected in an aqueous polymer two-phase system as outlined in a separate communication [35].

Results summary

Maps of adenovirus RNA sequences present in the nucleus and in the cytoplasm before the onset of DNA replication

Early after adenovirus infection only about 1% of the newly synthesized RNA in the nucleus is of viral origin. We have separated the strands of intact adenovirus DNA and several restriction enzyme fragments for hybridization analysis. ^{32}P-labeled complement specific DNA was incubated with saturating amounts of RNA and the fraction of each fragment strand represented in early nuclear or cytoplasmic RNA was determined by chromatography on hydroxylapatite.

The results show that early nuclear RNA saturates about 50% of the l-strand and 70% of the h-strand of the adenovirus DNA, suggesting that symmetric transcription occurs. When the complementary strands of several restriction enzyme fragments were analyzed 50–80% of both strands of all fragments were saturated by early nuclear RNA (fig.1). Thus large fractions of both strands are transcribed before the onset of DNA replication. Analysis of pulse-labeled nuclear RNA under denaturing conditions have not revealed RNA molecules larger than 45 S (unpublished) even after short pulses, indicating that the large transcripts, if present, are rapidly processed. The early mRNA sequences in the cytoplasm are derived from 25% of the l-strand and 15% of the h-strand of ad2 DNA. They are located in four separated regions, two on each complementary strand, as shown in fig.1. The difference in sequence representation between nuclear and mRNA indicates that extensive processing may occur in order to generate mRNA from the nuclear RNA species. It is, however, difficult to establish this pathway since the amount of RNA, which is transported out to the cytoplasm, is only a fraction of the

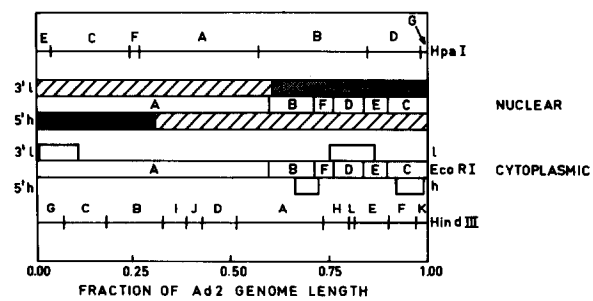

Fig.1. Maps of adenovirus RNA sequences present in the nucleus and in the cytoplasm before the onset of DNA replication. The map was deduced from results of liquid hybridizations between early nuclear or cytoplasmic RNA and separated strands of adenovirus DNA and several restriction enzyme fragments. Regions of the ad2 genome corresponding to mRNA sequences are indicated with unfilled bars. Nuclear RNA saturated 70% of the h- and 50% of the l-strand. These regions are indicated by striped bars extending inwards from the 3′-terminus of each strand. Since 50–80% of several fragment strands were saturated with nuclear RNA, most of both strands are probably expressed in nuclear RNA as indicated by stippled bars. The map of early nuclear RNA sequences is only tentative at present. The assignment of the 5′- and 3′-end stems from data reported by Sharp et al. [19].

total amount synthesized in the nucleus. It therefore remains to be established whether early mRNAs are cleavage products from large transcripts or whether they are the end products of a minor and undetected pathway of RNA synthesis.

Maps of adenovirus RNA sequences present in the nucleus and the cytoplasm late after infection

Late after adenovirus infection about 40% of the newly synthesized nuclear RNA is of viral origin and the remaining RNA consists primarily of host-cell ribosomal RNA sequences. Late nuclear RNA saturates about 85% of the l-strand and 10–15% of the h-strand whereas late cytoplasmic RNA saturates 65–70% of the l-strand and 25% of the h-strand [22]. Liquid hybridization analysis of late nuclear RNA has shown that the nucleus contains RNA sequences which are complementary to mRNA in the cytoplasm and overlapping sequences are synthesized at least from fragments *Eco*RI-B and *Eco*RI-C [19,22]. Double-stranded RNA with a complexity corresponding to more than 60% of the ad2 genome can be detected late after infection [19,22]. However, only a minor fraction of the newly synthesized RNA can be obtained in double-stranded form which indicates that late transcription is not symmetric in the sense that RNA is transcribed from both strands simultaneously with an equal frequency. Although RNA sequences which saturate 25% of the h-strand are present in mRNA late after infection, nearly all mRNA which can be pulse-labeled 17–20 h after infection is complementary to the l-strand [22]. The same preference for the l-strand late after infection has been demonstrated when RNA synthesis is carried out in vitro in isolated nuclei.

Our results thus indicate that all or nearly all mRNA sequences which are synthesized late after infection are derived from the l-strand although sequences can be detected in the cytoplasm which saturate 25% of the h-strand. This observation suggests that mRNA which is complementary to the h-strand is primarily transcribed early and persists until late after infection. Thus the switch from early to late transcription may include termination of h-strand transcription. Messenger-RNA sequences which are present exclusively late after infection include sequences which are complementary to the h-strand (see below). This result is puzzling in view of our finding that few sequences are transcribed late which are complementary to the h-strand. Conceivable explanations for this observation include the possibility that these sequences are synthesized early and retained in the nucleus until late after infection. Alternatively these sequences are transcribed with a much lower frequency than l-strand sequences and therefore escape detection.

The mRNA sequences which are present exclusively late hybridize to several regions which are scattered along the entire genome (fig.2). A major block containing late genes is located between positions 0.25 and 0.65 on the unit map of the ad2 genome. Three additional minor blocks are detected on the l-strand. Messenger-RNA sequences present exclusively late and complementary to the h-strand of the viral DNA appear to originate from two separate blocks located within fragments *Hind*III-B and *Hind*III-C respectively (fig.2). Other investigators have obtained similar maps for early and late viral mRNA although minor differences must still be resolved concerning the exact location of the mRNA sequences [36,37].

Purification and in vitro translation of complement specific adenovirus mRNA

An aqueous polymer phase system containing 6.3% (w/w) Dextran and 3.5% (w/w) polythylene glycol in 10 mM phosphate buffer, pH 8.0, was introduced to select RNA–DNA hybrids from unhybridized RNA

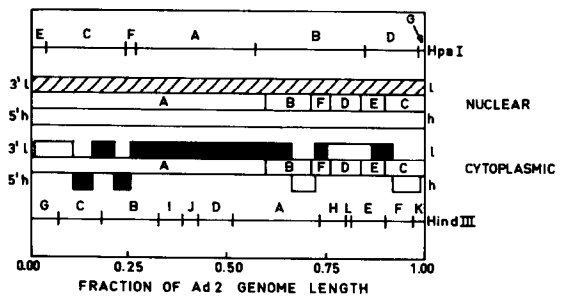

Fig.2. Maps of adenovirus RNA sequences expressed in the nucleus and present in the cytoplasm late after infection. The map was constructed as indicated in fig.1. Regions corresponding to mRNA sequences exclusively present late after infection are indicated with filled bars and regions corresponding to early mRNA sequences are indicated with unfilled bars. The nuclear RNA sequences derived from the l-strand are indicated with striped bars. Nuclear RNA sequences derived from the h-strand are not shown since they probably represent persisting RNA which was synthesized early after infection.

[35]. The top phase of this system contains DNA and RNA–DNA hybrids whereas more than 99% of the unhybridized RNA is confined to the bottom phase. After phase separation the hybrids were melted by heat and fractionated by oligo(dT)–cellulose chromatography. Under these conditions the polymers and the DNA percolate and only the polyadenylated mRNA absorbs to the column. In this way specific mRNA can be recovered with a 50–90% purity depending on the concentration of viral sequences in the starting material. In order to assign the genes for different viral polypeptides to the complementary strands of ad2 DNA, early and late viral mRNA was selected on the strands of adenovirus DNA according to this procedure. The isolated mRNA was readily translated in vitro in a wheat-embryo extract. Early mRNA selected on the h-strand directed the synthesis of polypeptides E72K, E19K and E11K. In contrast, early mRNA selected on the l-strand, directed the synthesis of five different polypeptides referred to as E44K, E42K, E40K, E16K and E12.5K. It has previously been demonstrated that all these polypeptides are induced early during adenovirus infection [38–40]. Independent proof of their viral origin has been obtained by other investigators [32] who have demonstrated the synthesis of peptides of similar size after selection of early mRNA on fragments of adenovirus DNA by urea–hydroxylapatite chromatography. Late mRNA has been found to code for several polypeptides most of which are derived from the l-strand. After selection of late mRNA sequences on the h-strand only three polypeptides could be observed which were absent when extracts were incubated with RNA from mock-infected cells. These polypeptides correspond in size to polypeptides IVa_1, IVa_2 and IX, all previously identified as structural proteins [41]. Since both early and late viral mRNA has been selected on restriction enzyme fragments of adenovirus type 2 DNA by other investi-

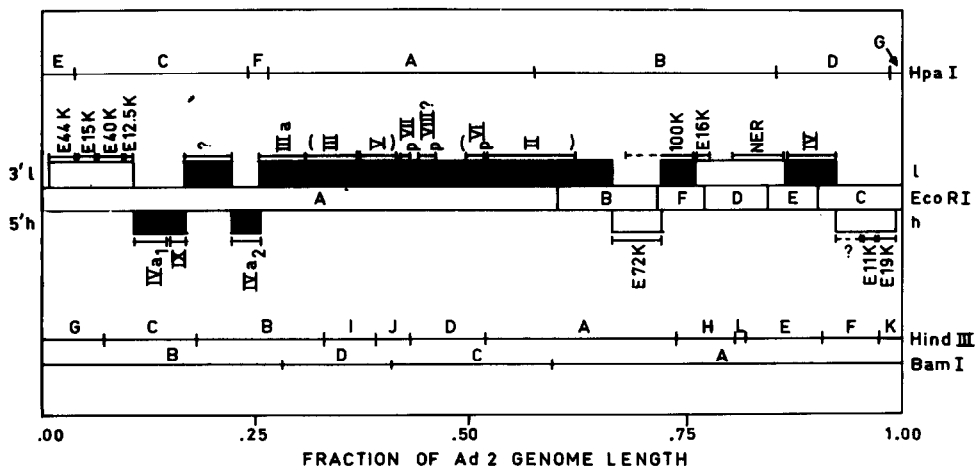

Fig.3. A preliminary map of some virus specific polypeptides which are synthesized early and late during productive infection with adenovirus type 2. Early and late polypeptides are designated as described [31,35,40]. The maps of early and late mRNA [30] were used as the basis for this figure. The unfilled and filled bars represent early and late mRNA sequences, respectively. Assignment of early and late polypeptides to several restriction enzyme fragments of ad2 DNA has been reported by the Cold Spring Harbor group [31,32,42]. The strand assignment of some polypeptides is reported here. The region designated NER within the *Eco*RI-D fragment is probably a non-essential region (NER) [30,43] since these sequences are deleted in some SV40-adenovirus hybrid viruses [44]. The positions of early products on the l-strand are based on the fragment assignments of E44K, E15K and E16K by Lewis et al. [32] and the strand assignment reported here. The positions of E40K and E12.5K are only tentative and based on the size and the number of mRNA species recovered from the left hand region [45]. The positions of the early products on the h-strand agree both with the fragment [32] and our strand assignments. The positions of late products on the l-strand are based on the fragment assignments reported from Cold Spring Harbor Laboratory [31,42]. The relative orders of the genes for polypeptides III and V as well as II and pVI are still undetermined and the position op pVIII is uncertain [31,42]. The positions of the late products derived from the h-strand agree with the fragment assignment of IVa_2 relative to IIIa [42] but the relative order of IVa_1, IX and IVa_2 is unclear.

gators [31,32] it is now possible to combine all information and provide a tentative map of several gene products of adenovirus DNA. Figure 3 shows an attempt to compile a gene-product map of adenovirus DNA. Some map positions are firm but most of them are only suggestive. It remains to verify the identity of the polypeptides synthesized in vivo and in vitro and to select mRNA on the strands of specific fragments. In the meantime the map may be useful to illustrate the complexity of gene expression during adenovirus infection.

Synthesis of low molecular weight RNA species in adenovirus infected cells

Adenovirus infection leads to the production of large quantities of a virus coded 5.5 S RNA. An additional species of virus coded low molecular weight RNA (5.2 S) which migrates slightly faster on polyacrylamide gels than the well-characterized adenovirus specific 5.5 S RNA, has been isolated from cells infected with adenovirus type 2. Hybridization competition experiments and finger-print analysis of T_1 oligonucleotides indicate that the two species of virus coded low molecular weight RNA differ in their primary structures. The gene for 5.2 S RNA is located rightwards from the gene for 5.5 S RNA on the l-strand of the *Hin*dIII-B fragments of ad2 DNA and both RNAs originate between positions 0.29 and 0.32 on the unit map of ad2 DNA (fig.4). Both 5.5 S and 5.2 S RNA can be detected early after infection and in the presence of cytosine arabinoside or cycloheximide. After the onset of viral DNA replication the synthesis of 5.2 S RNA levels off whereas 5.5 S RNA is synthesized in increasing amounts. Both species are synthesized in isolated nuclei by an enzyme which resembles RNA polymerase III by its sensitivity to α-amanitin. In addition both RNAs are labeled with β-^{32}P-labeled GTP which suggests that they are initiated at separate promoter sites.

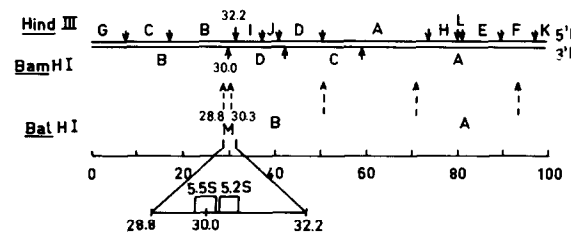

Fig.4. Location of the genes for two species of low molecular weight RNA on the adenovirus type 2 genome. The cleavage sites for restriction endonucleases *Hin*dIII and *Bam*HI are shown in the figure as well as selected cleavage sites for endonuclease *Bal*HI. The cleavage map is based on data which were kindly provided by Dr R. Roberts and his colleagues at the Cold Spring Harbor Laboratory. The 5.5 S and 5.2 S RNA species map between positions 0.29 and 0.32 on the unit map of ad2 DNA. The gene for 5.2 S RNA maps rightwards of the gene for 5.5 S RNA, but the exact distance between the genes is not known at present. Modified from ref.[33].

Discussion

The complex organization of the ad2 genome and the extensive transcription of the viral DNA, including sequences complementary to mRNA species [19,22] emphasize the role of post-transcriptional controls in the selection of specific mRNA species from a broader population of nuclear transcripts. However, exactly as in uninfected cells only a small fraction of the nuclear RNA is transported to the cytoplasm and therefore sequences which are destined to become mRNA may be derived from a special population of nuclear RNA molecules. The observation that the synthesis of some but not all early mRNA sequences is turned off late after infection suggests that a negative control of transcription exists. Thus there are obviously several ways in which the viral genome can modify and exploit the regulatory mechanisms of gene expression in the infected cell. Additional experiments are clearly needed to establish the importance of post-transcriptional cleavage of the nascent transcripts. Hopefully, this problem could be explored by mapping studies of transcripts which contain tetraphosphates at the 5′-ends.

Adenovirus specific 5.5 S and 5.2 S RNAs offer some interesting possibilities to study transcriptional regulation in an animal virus system. Since both RNA species apparently contain tetraphosphates at their 5′-ends and since the nucleotide sequence of the entire 5.5 S RNA is known [12] it may become possible to locate and determine the sequence of a promoter for mammalian RNA polymerase III.

With information now available on the physical location of DNA sequences destined to be represented in nuclear RNA, mRNA, 5.5 S and 5.2 S RNA, efforts will be directed towards a structural analysis of the

regions which are expected to play a role in the regulation of viral genome expression.

Information on the exact localization of genes for several structural proteins is now rapidly accumulating. Thus it will soon be possible to study how the synthesis of individual polypeptides is regulated. Since the mRNA sequences expressed in transformed cells have already been mapped on the genome [24], the gene-product map may also aid to identify the proteins required for induction and maintenance of transformation.

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