Volume 10 Number 4 1982 Nucleic Acids Research

The orderly splicing of the first three leaders of the adenovirus-2 major late transcript

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Received 7 December 1981; Revised and Accepted 27 January 1982

ABSTRACT

A strategy based on the hybridization of labeled nucleoplasmic RNA to a short cloned cDNA probe was devised to study the ligation of the three first leader sequences (Le1, Le2, Le3) of the major late adenovirus-2 transcript. The hybridized RNA was subsequently fractionated by electrophoresis and identified with the aid of restriction fragments of the DNA probe. The ligations were shown to occur stepwise and in an orderly fashion. Le1 and Le2 were first ligated without detectable lag time. The tripartite leader was formed after a lag time of 10-15 min probably due, for a large part, to the stepwise excision of the intervening sequence between Le2 and Le3. The possible processing intermediate Le2-Le3 was not detected.

INTRODUCTION

Since the discovery that most genes contain intervening sequences (IVS) interrupting the coding sequences (for review, see 1,2), many studies were focused on the characterization of the primary transcripts and of the maturation intermediates from defined premRNA (2). Experimentally, the hybrids of nuclear steady-state RNA and genomic DNA were analyzed either by the S1 nuclease mapping method (3) or by electron microscopy (4,5,6). The structure of processing intermediates was thus determined and it was also shown that certain individual IVS were excised in several steps (4, 7-9). The kinetics of formation of intermediates was studied in a few cases (10,11,12). Based on the structure and relative frequency of processing intermediates, preferential pathways for excision and ligation were proposed (6,7,9,11), but contradictory results were obtained for the globin gene (7,9). In fact, in many cases, a large number of IVS and the possible existence of excision intermediates for indivi-

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dual IVS may complicate the situation so that the chronology of excisions and ligations is difficult to establish.

To determine whether the excision of IVS occurs in an orderly fashion, we choose to study the kinetics of splicing of the three first leaders of the major late adenovirus-2 (Ad2) transcript. The splicing of the three leaders (41,71 and 88 nucleotides) separated by two IVS of 1000 and 2350 nucleotides gives birth to a tripartite leader sequence of 200 nucleotides which is bound to more than a dozen different mRNA bodies (13,14). Hybridization of nucleoplasmic RNA pulse-labeled for various times to a DNA probe containing the spliced leaders allowed us to follow the formation of processing intermediates and to determine the chronology of ligation of the three leader sequences.

MATERIAL AND METHODS

Viral infection and RNA labeling

Hela cells were grown in suspension culture, infected with adenovirus-2 (100 plaque-forming units per cell) and harvested at 16 h post-infection. Labeling was with [^{3}H]-uridine (10 Ci/mmol) at a cell concentration of 6 x 10^6 cells/ml (15) in the last minutes of infection. In order to obtain equivalent specific activities of nuclear RNA in the kinetics experiments, various amounts of [^{3}H]-uridine were used : 500 µCi/ml (10 min), 300 µCi/ml (15 and 25 min), 200 µCi/ml (40 min) and 150 µCi/ml (75 mn). Incorporation was halted by pouring the cells onto an equal volume of crushed frozen phosphate buffer-saline.

Preparation of nuclei and nuclear RNA

Labeled infected cells were resuspended in an isotonic buffer derived from that of Mory and Gefter (16) (20 mM Hepes, pH 7.4, 25 mM KCl, 3 mM $CaCl_2$, 2 mM Mg $(CH_3COO)_2$, 2 mM dithiothreitol, 0.3 M sucrose containing 0.05% Triton X-100). After 10 min, the cells were broken in a Dounce homogenizer until no more than one unbroken cell per 100 nuclei persisted. The suspension was centrifuged at low speed on a 30% sucrose cushion made in the isotonic buffer without detergent. The non-sedimented fraction was used as the source of cytoplasmic RNA after clarification by centrifugation (15,000 g, 10 min). The nuclear pellet

was resuspended at 2-3 x 10^7 nuclei/ml in RSB (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$). The nuclei were broken by ultrasonication (15) and the saline concentration was adjusted to 0.5 M NaCl and 10 mM $MgCl_2$. After an RNAse-free DNAse treatment, the suspension was centrifuged on a 15-30% sucrose gradient (17). The nucleoli were found in the pellet and the nucleoplasmic material containing hnRNA, along the gradient. RNA was phenol-extracted from the cytoplasmic and nucleoplasmic fractions (15) and was further purified by DNAse treatment and chromatography on Sephadex G75 (18).

Cloning procedure

Cloning strategy was based on the method of Zain et al. (19). mRNA-cDNA hybrid was prepared by reverse transcription of cytoplasmic poly(A)$^+$ RNA from late adenovirus infected cells (20). The hybrid molecules were tailed with dC using terminal transferase and annealed to pBR322 DNA which was elongated at the PstI site with dG (21). The plasmids were first selected for their ability to transform the EK2 **E.coli** strain DP50 to tetracycline resistance (20). Colonies containing the 2 first adenovirus late leader sequences were then identified using a modification (22) of the **in situ** colony hybridization procedure of Grunstein and Hogness (23). The [^{32}P]-labeled XhoI-F fragment of Ad2 DNA (15.5 - 22.0 map units, see Fig. 1A) served as a probe. The selected transformed bacteria were grown at 37°C in a medium supplemented with diaminopimelate, thymidine and tetracycline and amplified by adding chloramphenicol (20). Extraction and purification of plasmid DNA were achieved by the cleared lysate technique of Clewell et al. (24) followed by ethidium bromide-CsCl equilibrium gradient. DNA inserts were prepared by PstI cleavage and purified in neutral sucrose gradients.

Restriction nuclease cleavage and gel electrophoresis.

Restriction endonucleases were purchased from New England Biolabs, and Boehringer. Cleavage reactions of plasmid and insert DNAs were carried out under the conditions recommended by the suppliers. Analytical separation of endonuclease fragments was made by agarose (2 or 2.5%) or polyacrylamide (5 or 8%) gel electrophoresis. The bands were stained with ethidium bromide,

visualized over a long wave UV-illuminator and photographed.

Hybridization methods and isolation of the hybridized RNA.

5-10 μg of nucleoplasmic RNA (about 6-12 x 10^5 cpm) or cytoplasmic RNA was mixed with 150-300 ng of the DNA probe containing the leader sequences. The DNA excess was at least 3-5 fold. The mixture was ethanol-precipitated and dissolved in 30 μl of 80% formamide, 0.4 M NaCl, 0.04 M Pipes, pH 6.4, 1 mM EDTA (25) and denatured at 75°C for 10 min. Samples were allowed to hybridize for 3 h at 55 or 59°C. After hybridization, they were diluted in 0.5 ml of 10 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 5 mM EDTA and the non-hybridized RNA was digested with 2 μg of RNAse A and 10 U of RNAse T1 (30 min, 37°C). The enzymes were digested with 50 μg of proteinase K in the same conditions. The double-stranded nucleic acids were phenol-extracted, precipitated with ethanol, resuspended in 30 μl of TBE (50 mM Tris-borate buffer, pH 8.3, 1 mM EDTA) containing 70% formamide and 10% glycerol and heated at 70°C for 10 min. The material was immediately electrophoresed in 12% polyacrylamide slab gels cast in TBE containing 8.3 M urea. Fluorography was according to Laskey et al. (26).

RESULTS

Selection of a cloned DNA probe. Its restriction map.

For detection of the ligation of the leader sequences from the major late adenovirus transcript (Fig. 1A), a DNA probe containing the tripartite leader sequence was required. The bulk of late cytoplasmic RNA was used for the preparation of mRNA-cDNA hybrids which were cloned in pBR322 as detailed in Material and Methods. Clones were selected for the presence of the two first leaders and three of them were further analyzed by restriction enzyme cleavage. The 435 base pair long sequence inserted in clone 2 fitted best our purpose. All of the selected enzyme sites present in the DNA of the tripartite leader (19,27) were detected at the expected positions (Fig. 1B). Thus, clone 2 DNA contained the leader sequences from at least position 5 (MboII site) up to position 173 (XhoI site). The presence of an extra-leader sequence (unidentified yet) at the 3' end of the cloned DNA indicated that Le3 extended up to position 200. We did not verify that Le1 extended up to position 1, since the

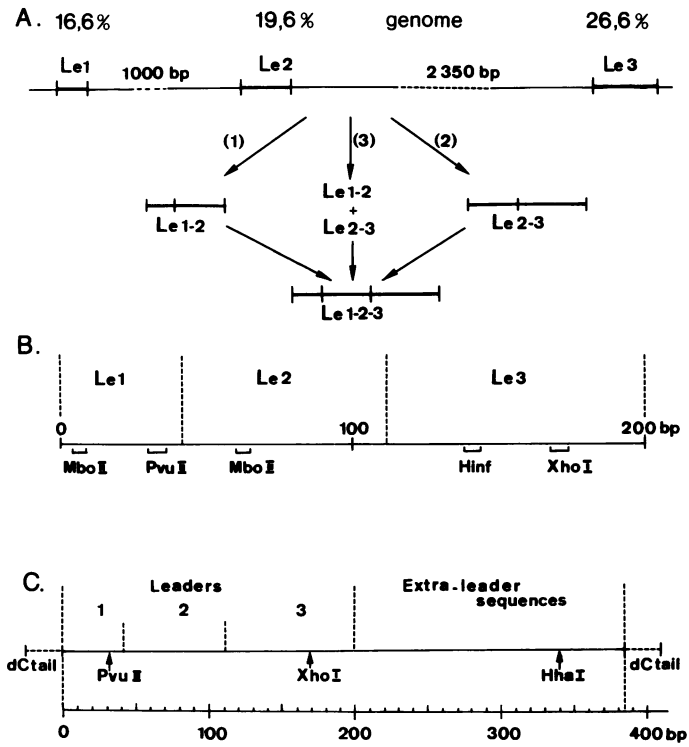

Fig. 1 : A) Map of the adenovirus genome depicting the leader region of the major late transcription unit (14) and possible pathways for the splicing of the leader sequences. The 3 leaders (41,71 and 88 nucleotides long) are in the 5' part of the long premRNA initiated at 16.6% on the genome. Le1-2, Le2-3 represent possible intermediates and Le1-2-3 the tripartite leader sequence. B) Restriction enzyme map of the DNA corresponding to the tripartite leader sequence (according to Zain et al., 19). C) Schematic representation of the Ad2 sequences of clone 2. The intact DNA, or the DNA cleaved by restriction enzymes as indicated was used for hybridization to RNA.

presence or absence of 4 nucleotides would be of little consequence for our experiments.

The overall structure of the Ad2 DNA of clone 2 is given in Fig. 1C. From the MboII digestion data (not shown), we could calculate that the 5' dC-dG tail was 25 base pairs long. Assuming that a tail of similar size is present at the 3' end, this would indicate a 185 base pair long extra-leader sequence. A HhaI site was found to be located at position 340 from the 5'

end of the Ad2 DNA. Using the restriction enzymes HhaI, BstNI and HphI, we showed that the extra-leader sequence was not the x, y and z leaders (28,29) nor the fiber message (19). The HhaI, PvuII or XhoI-cleaved DNA were used as probes (without separation of the fragments) in addition to the intact Ad2 cloned DNA.

Detection of cytoplasmic leader sequences by the DNA probe.

At high formamide concentration, difficulties may be encountered in the association of DNA with short RNA sequences such as the Ad2 leader sequences (30). In addition, the temperature window allowing the RNA-DNA association may be very narrow (30). The following experiments were performed in order to determine the optimal hybridization conditions and to characterize the spliced leader sequences.

The specificity of the assay was determined by using a 3h-labeled cytoplasmic RNA from cells at the late period of infection, when the tripartite leader was present at high frequency. Very little if any self-annealing RNA was observed in cytoplasmic RNA (Fig. 2A, lane a). A predominant RNA sequence of about 200 nucleotides, in all likelyhood the tripartite leader sequence was protected by the intact clone 2-Ad2 DNA (Fig. 2A, lanes b and c). The XhoI DNA fragment from the 5' end to position 170 protected a RNA sequence of about 170 nucleotides (lane d). The DNA probe uncompletely cleaved by PvuII (leader sequence between positions 33 and 200) protected predominantly a RNA of about 165 nucleotides (lanes e and f) and accessorily the 200 nucleotide long sequence identical to that found in lanes b and c. When the PvuII DNA fragments were further cleaved by the XhoI enzyme (lane g), a major RNA band of about 140 nucleotides was detected (corresponding to the PvuII-XhoI fragment, positions 33-170) in addition to a minor band of 170 nucleotides with the same mobility as that shown in lane c. Additional faint bands were occasionally observed which were not analyzed but which may arise from the presence of extra-leader sequences in the DNA probe (see also next section).

From these results, we conclude that our conditions of hybridization allow the specific detection of the 200 nucleotide long tripartite leader sequence and of derived fragments of

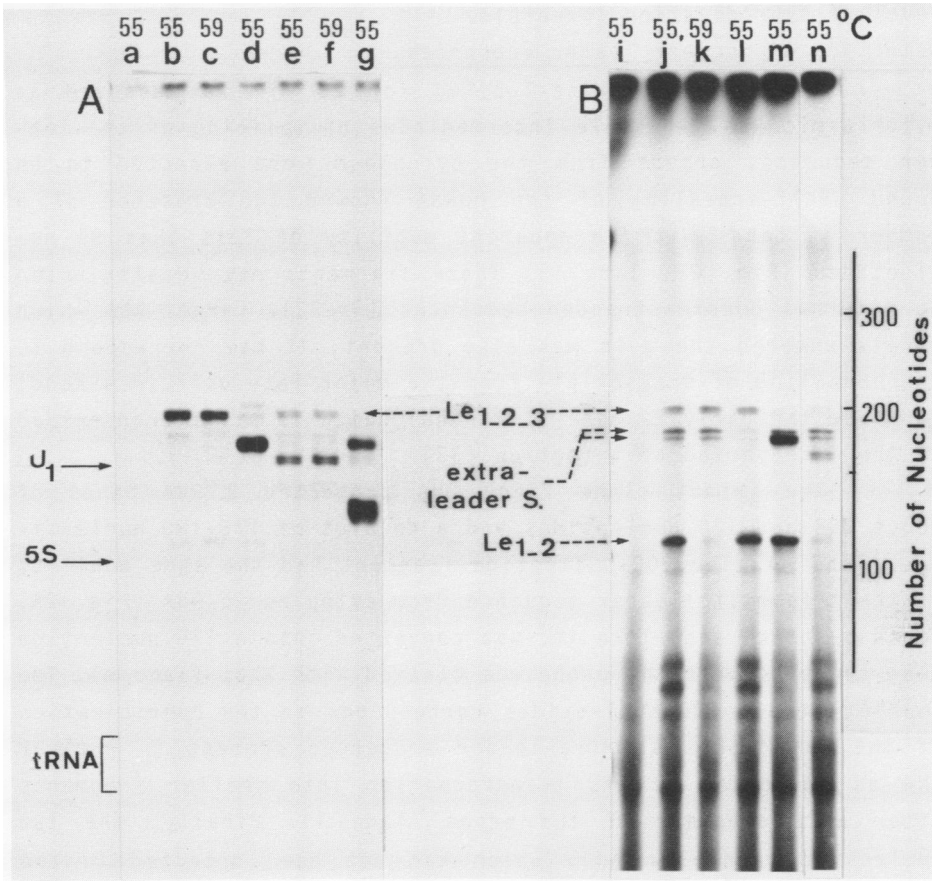

Fig.2: Electrophoretic analysis of the RNA sequences hybridized to the Ad2 DNA from clone 2. Cytoplamic RNA isolated from 3h-labeled infected cells (A) and nuclear RNA isolated from 20-min labeled infected cells (B) were hybridized to the DNA probe at 55 or 59°C as indicated. The hybridized RNA was analyzed in 12% polyacrylamide slab gel. Hybridization : a and i, controls without DNA; b, c, j, and k, intact probe; d and m, XhoI-cleaved DNA; e, f and n, PvuII-cleaved DNA; g, XhoI and PvuII-cleaved DNA; l, HhaI-cleaved DNA. The scale (number of nucleotides) is calculated from the length of the RNA sequences, as expected from the DNA sequence data. A mixture of small RNA (U1, 5S and tRNA) served as markers.

smaller size. The results were similar when hybridization was performed at 55 and 59°C. An unexplained observation is that the removal of the 30 nucleotides from the 3' end of the tripartite leader from the DNA probe (XhoI fragments, lane d) enhanced the

level of hybridization threefold.

Detection of nuclear leader sequences.

Nuclear RNA, pulse-labeled for 20 min was assayed as cytoplasmic RNA. Possible intermediates of splicing of the leader sequences absent from the cytoplasm were expected to be found. Self-annealing of the hnRNA showed the presence of a number of bands with an apparent mobility of less than 85 nucleotides (Fig. 2B, lane i). These fragments may be attributed to internal double-stranded sequences (31-32). Larger RNA which barely entered the gels was also present. It may correspond to the annealing of transcripts from the major late unit (r strand) and from the early E2 gene (l strand) which is still transcribed at the late period of infection (33).

The intact clone 2-Ad2 DNA protected 2 RNA bands of about 200 and 120 nucleotides and a doublet of 175-180 nucleotides (lane j). The 200 nucleotide fragment had the same mobility as the tripartite leader sequence from cytoplasmic RNA (Fig. 2A, lanes b and c) and, like it, was converted into a 170 nucleotide long sequence when the probe was cleaved with XhoI (lane m). The doublet at 175-180 nucleotides corresponds to the hybridization of the DNA probe to extra-leader sequences present in nuclear RNA as indicated by its transformation into smaller fragments after HhaI cleavage of the probe (lane l). Finally, the 120 nucleotide band (lane j), which had not been detected in the cytoplasm, was absent when the nuclear RNA was annealed with the PvuII-cleaved probe (lane n). Thus, it corresponds to the Le1-2 intermediate of splicing whose size is 112 nucleotides. In contrast to all other RNA fragments whose presence and relative amount did not depend on the annealing temperature (between 55 and 59°C), the Le1-2 intermediate hybridized 10 times less at 59°C than at 55°C (lanes j and k).

Thus, hybridization of a 20 min pulse-labeled nuclear RNA to the DNA probe allowed the clear detection of the tripartite leader Le1-2-3 and of the intermediate Le1-2 (200 and 112 nucleotides) but not of the 159 nucleotide long Le2-3 putative intermediate (Fig. 1A). It is improbable that this is due to inefficient hybridization conditions since the PvuII fragment (positions 33-200) which is very similar to the Le2-3 leader

(positions 41-200) was well detected (Fig. 2A, lanes e, f). Since a band with an intensity 50 times lower than that of the major bands may be detected under our experimental conditions and since no band was found at the expected position, we conclude to the absence of the Le2-3 intermediate in nuclear RNA.

No well-defined band with the length of the intact probe (385 nucleotides) was detected neither in the cytoplasm nor in the nucleus. This indicates that the splicing of the tripartite leader to the extra-leader sequence detected by our probe was not a frequent event.

Kinetics of ligation of the leader sequences

To follow the appearance of the spliced leaders, we studied the nuclear RNA from infected cells after various periods of labeling between 10 min, time allowing a nearly maximal incorporation into the initial transcripts and 75 min, when steady-state labeling is reached (12). In order to eliminate variations due to the labeling kinetics of the nucleotide pool and of the primary transcripts, and to study only the splicing reactions, we used RNA samples with identical amounts of radioactivity for each hybridization reaction. The amount of radioactive viral RNA in the samples was henceforth identical, since the distribution of the label between cellular and viral RNA does not change up to 120 min (15, 34). The possible interferences due to widely different RNA amounts were eliminated by using adequate amounts of [^{3}H]-uridine for labeling as described under Material and Methods.

To distinguish between the leader and extra-leader sequences which may be recognized by Ad2 cloned DNA, we used a Xho-I cleaved probe (Fig. 3A) and a XhoI, HhaI-cleaved probe (Fig. 3B). Nucleoplasmic RNA (6-10 x 10^5 cpm) was hybridized to an excess of DNA. Beside the self-annealing RNA of small or large size (see preceeding section), we found 3 bands for each DNA probe. Two major bands had the same mobility with both probes. They correspond to the Le1-2 and Le1-2-3 sequences truncated after XhoI cleavage of the probe (as in Fig. 2, lane m). The minor band found at 180 nucleotides after XhoI cleavage (Fig.3A) contains an extra-leader sequence since its size was reduced to 130 nucleotides after HhaI, XhoI cleavage of the probe (Fig.

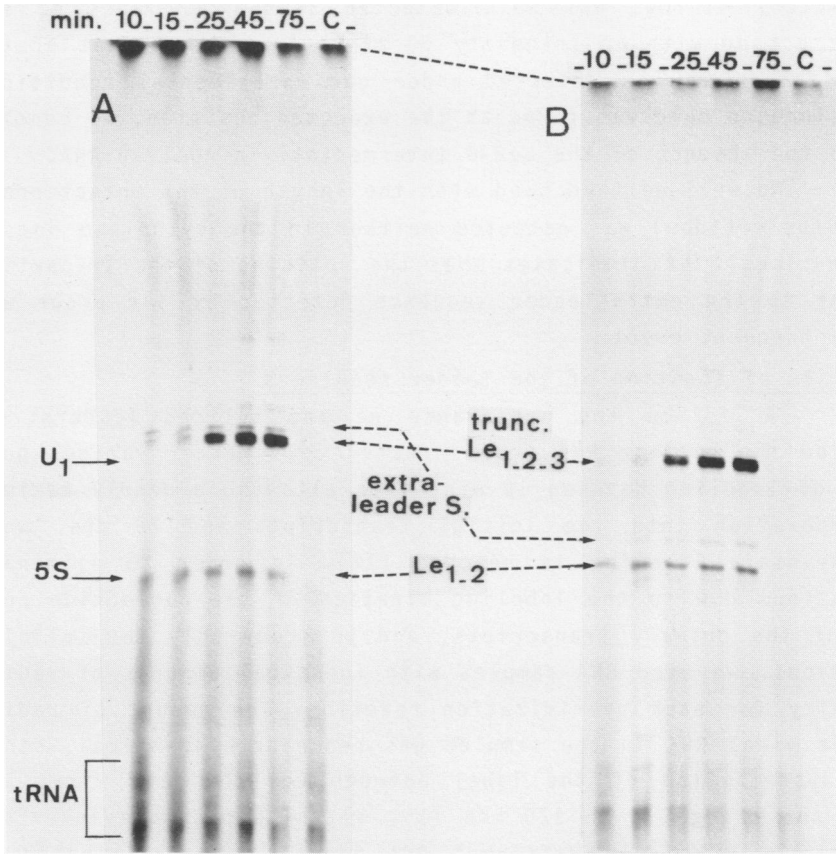

Fig.3: Formation of ligated leader sequences. Nucleoplasmic RNA was isolated from infected cells labeled for various periods as indicated. RNA samples containing $6x10^5$ cpm were hybridized to the same excess of XhoI-cleaved DNA (200 ng) (A) or of XhoI, HhaI-cleaved DNA (B). After such cleavages, the tripartite leader is recovered as a 170 nucleotide long fragment (see also Fig. 2, d and m). Lanes denoted C represent controls without DNA for 25 min labeled RNA.

3B). The length of the fragments indicates that they contain extra-leader sequences only and that the XhoI 3' end of Le3 was absent (Fig. 1). No RNA fragment of 210 (A) and 170 nucleotides (B) which would suggest a splicing of the tripartite leader to the extra-leader sequence was detected. On the other hand, in agreement with the results of Fig. 2B, we did not observe any band representing Le2-3 at any time of labeling.

The examination of the gels shows that Le1-2 is already detected with nearly maximal incorporation at 15 min whereas Le1-2-3 is well labeled at 25 min only and reaches its maximum intensity between 45 and 75 min. The curves of Fig. 4 were plotted on the basis of the densitometric analysis of the autoradiograms from 4 experiments. We observe that Le1 and Le2 are spliced without any detectable lag time and the plateau is already reached after 15 min. Thus, the junction of the two first leaders is an early event of the processing of the major late adenovirus transcripts. In contrast, the tripartite leader is made after a lag time of about 12 min, the maximal rate of formation is between 15 and 25 min and the plateau is reached only after about 60 min. As no Le2-3 intermediate was detected, we may assume that Le1-2 is an obligatory intermediate for the formation of Le1-2-3. However, the long lag time excludes that

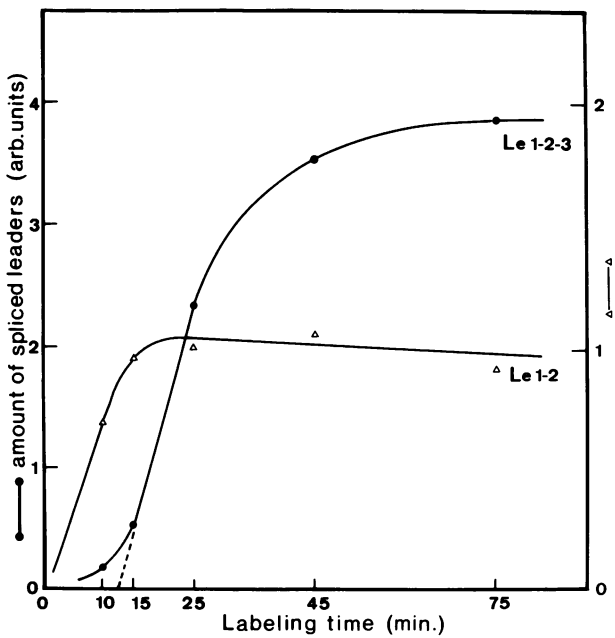

Fig.4: Kinetics of ligation of leader sequences. The amount of Le1-2 and Le1-2-3 was estimated by planimetry from recordings of gels similar to those of Fig. 3. The results are given in arbitrary units. The scale of ordinates for Le1-2-3 is twice that used for Le1-2.

the formation of Le1-2 is the only prerequisite to that of Le1-2-3. It remains that the ligation of the leaders of the major late adenovirus transcript is a sequential event and that pathway 1 (Fig. 1A) is the only one occuring **in vivo**.

The unidentified extra-leader sequences protected by the DNA probe in Fig. 3 correspond to these particular sequences present in primary or partially processed transcripts and not yet ligated to the leaders. Maximal intensity of the bands (Fig. 3A and B) was already reached after 15 min. This probably reflects the rapid overall equilibration of labeling of the various viral sequences in infected cells and makes the delayed ligation of the third leader even more striking.

At steady-state, only 20-25% of the spliced leader sequences as detected by our probe was found in the Le1-2 intermediate, the rest being in the tripartite leader. This is compatible with the idea that Le1-2 is an early intermediate with a short half-life whereas the tripartite leader which is part of numerous premessenger RNA molecules in the course of processing has a longer half-life in the nucleus.

DISCUSSION

Splicing of premessenger RNA has been shown to occur shortly after polyadenylation (6, 7, 35). Though preferential pathways of excision of IVS have been proposed (6, 7, 9, 11), the situation remains complex and no general rule concerning pathways of excision-ligation can be inferred from the available data. We propose here another strategy based on the hybridization of **in vivo** labeled RNA to a DNA probe containing a small number of splice junctions. The use of labeled RNA is advantageous as compared to steady-state RNA as it allows kinetic studies and may permit the detection of rapidly turning-over intermediates less easily detectable at steady-state. After hybridization to the DNA probe containing the spliced junction sequences, such sequences can be unambigously recognized, allowing to establish the chronology of formation of intermediates and the pathway of splicing events. The choice of DNA probes containing only small sets of neighbouring spliced junctions may avoid complications due to the presence of too many fragments in

subsequent analysis. DNA complementary to mature RNA sequences (or its restriction fragments) is the most convenient probe, its main disadvantage being that the detection of the excision intermediates for individual IVS is not possible. Such difficulty may certainly be overcome by the construction of suitable DNA probes. It remains that the method is of general application as long as nuclear RNA may be easily labeled **in vivo**. Such strategy might also be useful in the determination of ligations in **in vitro** assays.

This strategy was applied to the splicing of the leader sequences of the major late adenovirus transcripts. We demonstrated the formation of the intermediate Le1-2 as well as that of the tripartite leader Le1-2-3 but we could not detect the other possible intermediate Le2-3. These results are in general agreement with the data of Berget and Sharp (4). The ligation between Le1 and Le2 proceeded rapidly, without apparent lag time and occurred perhaps as early as or immediately after polyadenylation (36). The small amount of Le1-2 as compared to Le1-2-3 at steady-state strongly suggests that Le1-2 is a rapidly turning over processing intermediate. In contrast to Le1-2, the formation of the tripartite leader only occurred after a lag time of about 12 min. This lag time may be explained by the obligatory formation of Le1-2 (Fig. 4) and by that of several excision intermediates of the intervening sequence between Le2 and Le3 (4) prior to the formation of the tripartite leader. The quadripartite leader Le1-2-i-3 found in a small proportion of the cytoplasmic messenger RNA (37) might be one of these excision intermediates and its study might be of particular interest for the comprehension of splicing regulation.

The formation of the tripartite leader may precede or not its binding to the various mRNA bodies. Kinetics studies on the Ad2 fiber premRNA showed that processing intermediates of 11.5 and 6.3 x 10^3 nucleotides were made from the large primary transcript (23 x 10^3 nucleotides) with lag times of 10-20 min (12). This suggests that the excision of the IVS between Le1-2 and Le3, and between Le3 and the mRNA bodies may occur simultaneously and that the splicing of the leaders does not obligatorily precede that of the mRNA bodies.

Our studies show that the first splice to be made is the one located closest to the 5' end of the premessenger RNA. At the present time, we ignore if this is a general rule or a peculiarity of the processing of the adenovirus major late transcript.

ACKNOWLEDGEMENTS

This work was partially supported by grant CRL 80.1.040 (INSERM). We are grateful to Dr. M. Perricaudet, Dr. F. Gannon and Mr. J.M. Garnier for advice and help in cloning procedures. We thank Mr. G. Hildwein and Mrs K. Dott for their expert technical assistance.

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