Hybridization properties of sequences adjacent to triphosphorylated 5'-ends of nuclear pre-mRNA from mouse Ehrlich carcinoma

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ABSTRACT

Triphosphorylated 5'-end fragments about 100 nucleotides long were prepared from purified nuclear pre-mRNA using a modified hydroxyapatite method /1/. These fragments as well as fragments of total pre-mRNA of the same size were polyadenylated *in vitro* by ATP:RNA adenylyltransferase and used as templates for the synthesis of [³²P] cDNA by reverse transcriptase in the presence of an oligo(dT) primer. The use of cDNA transcribed from the triphosphorylated 5'-end fragments of pre-mRNA (5'-cDNA) and from the total pre-mRNA fragments allows one to calculate the complexity of the 5'-end fraction pre-mRNA and to detect these sequences in polysomal mRNA.

Sequences adjacent to 5'-phosphorylated ends of pre-mRNA represent a specific class of sequences with a complexity of about 200 kb.

It was also found that about 25% of total pre-mRNA and about a half of sequences adjacent to triphosphorylated 5'-ends are present in polysomal mRNA. A high homology between triphosphorylated 5'-end fragments of pre-mRNA and mRNA sequences may be explained in terms of splicing. Less than 30% of 5'-cDNA hybridized to moderately repetitive DNA while most of them are represented by unique DNA sequences. About 15% of 5'-cDNA contained oligo(dA) sequences originated from oligo(U) in pre-mRNA from which it was transcribed.

INTRODUCTION

Development of the recombinant DNA technology made it possible to work with individual eukaryotic genes. However, there are dozens of thousands of different genes in higher eukaryotes and it is difficult to extrapolate, to the whole genome, the data obtained for a particular, even rather large, group of genes. Therefore, experiments on individual genes should be supplemented with data obtained for the whole population of genes.

The aim of this work was to study the nature of starting sequences in the primary transcripts of pre-mRNA. The existence of triphosphorylated 5'-ends in nuclear pre-mRNA has been demonstrated earlier /1-4/. They may be considered, with some reservation, as the true beginnings of original transcripts in contrast to capped 5'-ends which are formed post-transcriptionally and do not necessarily correspond to the sites of initiation of transcription /5/. Thus, studies on triphosphorylated 5'-ends may give information about the fate of leader sequences in pre-mRNA.

Triphosphorylated 5'-ends of pre-mRNA from different tissues were detected as nucleoside tetraphosphates, pppGp and pppAp, formed after alkaline hydrolysis /1-4/. For further characterization of sequences adjacent to them, a new technique has been elaborated which allows one to prepare RNA fragments about 100 nucleotides long containing triphosphorylated 5'-ends. The method was based on hydroxyapatite fractionation of fragmented RNA.

Using this technique, the authors obtained more information about initial pre-mRNA sequences /6/. The main difficulty in that work was rather low specific activity of the in vivo labeled RNA, limited the possibilities for performing hybridization experiments. Moreover, direct hybridization of these fragments to mRNA was not possible while competition experiments are more complicated and more difficult for interpretation.

These difficulties have been overcome in the present work by getting cDNA copies of triphosphorylated 5'-end fragments. For this, the latter were polyadenylated by ATP:RNA adenylyltransferase and then transcribed by reverse transcriptase using oligo(dT) as a primer. Such a procedure results in obtaining a highly labeled and stable cDNA. The cDNA product synthesized is complementary to RNA and may be immediately tested in direct hybridization experiments with pre-mRNA and mRNA. It was another advantage of the procedure used.

The main result obtained was the demonstration of the complementarity of about half of cDNA transcribed from triphosphorylated 5'-end fragments (5'-cDNA) to polysomal mRNA. Thus,

about a half in initiation sequences in pre-mRNA seem to be conserved during the processing. A possible relation of this phenomenon to splicing is discussed.

MATERIALS AND METHODS

Cells and labeling. Ehrlich ascites carcinoma cells (10^8 cells) grown in mice were collected at the seventh day after transplantation. The ascitic fluid was removed by centrifugation, the cells were resuspended in 25-ml volumes of the Eagle medium and incubated for 45 min with a mixture of four ^3H nucleosides (2 mCi of each). In other experiments, non-labeled cells were used.

DNA was isolated by the phenol-detergent method. It was treated with RNAase and pronase, deproteinized additionally with SDS, phenol-chloroform, and precipitated with ethanol. For renaturation experiments, DNA was fragmented to 200 nucleotide segments by heating in 0.3 M NaOH for 30 min /7/.

mRNA was isolated according to a modified method of Staehelin & Shreier/8/. Ehrlich carcinoma cells were ruptured with 1% NP-40 in 50 mM tris-HCl (pH 8.0), 0.15 M KCl, 1 mM MgCl_2 , 0.25 M sucrose. The supernatant after centrifugation at 12,000 rpm for 15 min was ultracentrifuged in a discontinuous sucrose gradient (1 M and 2 M sucrose, in the same buffer, 7 ml of each in a SW-27 rotor for 17 hrs at 25,000 rpm. Polyosomes were collected from the bottom, RNA was deproteinized by treatment with SDS, phenol (pH 8) - chloroform, and precipitated.

Poly(A) $^+$ RNA was isolated using poly(U)-Sephadex chromatography /9/. The yield of poly(A) $^+$ RNA was of 0.5-1% of total polysomal RNA.

Pre-mRNA was prepared with the aid of the hot phenol fractionation procedure /10/ RNA fractions extracted at 65 and 85°C were collected, combined, treated with DNAase I (Whorthington, RNAase-free), and deproteinized additionally by treatment with phenol and chloroform.

The isolated pre-mRNA was fractionated by

ultracentrifugation in a 5-20% sucrose gradient in 0.05 M NaCl, 5 mM EDTA, 50 mM tris-HCl (pH 7.6), 0.25% SDS in a SW-27 rotor (Spinco) for 15-18 hrs at 17,000 rpm and 20°C. 28S and 18S ribosomal RNAs were used as markers. After centrifugation, pre-mRNA fractions heavier than 30S and 30-20S were collected, combined, and used in further experiments.

Fragmentation of pre-mRNA. To reduce the molecular weight of RNA, the latter was treated either with 0.1 N NaOH for 20 min at 4°C or with pancreatic RNAase, 0.05 µg/ml in 0.01 M phosphate buffer (pH 6.9), for 10 min at 4°C (RNAase was pre-boiled for 10 min in the buffer with 10 mM EGTA to destroy phosphatase traces). The samples were then neutralized (or treated with 1% SDS and phenol at 65°C for 15 min in the case of enzymatic fragmentation) and ultracentrifuged in a 5-20% sucrose gradient in SDS-buffer in a SW-41 rotor (Spinco) for 18 hrs at 36,000 rpm and 20°C.

After centrifugation, a narrow zone sedimenting at 4-5S (tRNA and 5S RNA were used as markers) was collected. More rapidly sedimenting fractions were also collected and the fragmentation was repeated until the bulk of RNA was recovered in the 4-5S peak.

Hydroxyapatite chromatography was performed according to Baiszar et al. /1/ with some modifications. Hydroxyapatite of DNA-grade (Bio-Rad, USA) was used. It was washed several times with 0.04 M sodium phosphate (pH 6.9) to remove small crystals. The column was packed (1 ml of gel per 200 µg RNA) and equilibrated with the same buffer. The RNA sample denatured by 80% DMSO treatment followed with ethanol precipitation was dissolved in 0.04 M sodium phosphate. RNA concentration should not exceed 200 µg/ml. The sample was applied to a column with a flow rate of 10 ml/hr. The elution was performed with 0.04-0.2 M sodium phosphate gradient (about 200 ml of the solution per 5 ml of the column). Once the main part of peak A containing internal RNA fragments came out (Fig. 1), the elution was continued with 50-75 ml of the buffer of the same concentration (usually 0.12 M). Then the elution was continued with a 0.12-0.4 M sodium phosphate gradient (100 ml) until peak B containing triphosphorylated 5'-end fragments contaminated

with internal ones appeared and came out. Peak B was collected, dialyzed against bidistilled water, and lyophilized. The second cycle of chromatography was performed in the same way. Now peak B consisted of almost only triphosphorylated 5'-end fragments.

To check the degree of enrichment, an aliquote of the material was hydrolyzed with alkali, chromatographed on DEAE-Sephadex, and the material with a negative charge of -5 to -6 was analyzed for phosphatase sensitivity as described previously /3/. Usually the yield of RNA in peak B was 0.5-1% of the total radioactive material, applied to hydroxyapatite.

Phosphatase treatment. Fragmented RNA contained 3'-phosphate groups to be removed before polyadenylation. For this purpose, the total fragmented RNA and the RNA of peak B (after a cycle of chromatography) were dialyzed against bidistilled water, lyophilized, and purified from traces of phosphate by gel filtration through Sephadex G-50 equilibrated with 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$. The RNA solution thus obtained was treated with alkaline phosphatase (BAPF, Worthington; purified from RNAase contaminations according to Wimmer /11/) in the same buffer. The reaction was stopped by adding EDTA to 5 mM. RNA was deproteinized by the phenol-chloroform (1:1) treatment, and the aqueous phase was extracted with distilled ethyl ether. RNA was then precipitated with ethanol in the presence of 0.2 M acetate, reprecipitated, and dried in vacuo.

Polyadenylation of RNA. ATP:RNA adenylyltransferase was isolated from *E.coli* according to Sippel /12/. It was free of RNAase activity: the sedimentation coefficients of ribosomal RNA after polyadenylation remained unchanged. Polyadenylation was performed in a medium containing 50 mM tris-HCl (pH 7.9), 10 mM $MgCl_2$, 2.5 mM $MnCl_2$, 0.25 M NaCl and 0.2mM ATP for 1 hr at 37°C. SDS (to 0.25%) and EDTA (to 5 mM) were added to stop the reaction and the mixture was heated for 10 min at 85°C. The efficiency of polyadenylation was checked by chromatography of RNA on oligo(dT) cellulose /13/. RNA was applied to a column in 0.8 M NaCl 0.05 M tris-HCl (pH 7.6), then the column was washed with 0.2 M NaCl, and bound RNA was eluted with water at 40°C.

Preparation of cDNA. The reaction medium contained 50 mM tris-HCl (pH 8.3), 6 mM $MgCl_2$, 4 mM DTT, 40 mM KCl, 0.5 mM dNTP, 20-25 μ Cl [^{32}P] dCTP (Amersham, 300-360 Ci/mmol) and actinomycin D (50 μ g/ml). A 50- μ l of the reaction mixture contained 0.5-1 μ g of RNA, 0.1-0.2 μ g of oligo dT₍₁₀₋₁₂₎ and 1-2 units of reverse transcriptase from AMV virus.

The mixture was incubated for 1 hr at 37°C. After deproteinization and alkaline hydrolysis of RNA, cDNA was purified by gel filtration through Sephadex G-50, precipitated with ethanol with a carrier *E.coli* tRNA, and dissolved in 50 mM NaCl, 50 mM tris-HCl (pH 7.6), 0.1% SDS.

Hybridization and renaturation were performed in solution (in 0.36-1 M NaCl). Reaction vessels contained either 50-100 μ g of DNA or 10-12 μ g of pre-mRNA or mRNA, and [^{32}P]cDNA (2000 cpm). The solution was placed either in sealed capillars or in glass tubes under a layer of mineral oil Bayol (Serva). Annealing was performed at 65-70°C (depending on salt concentration) for a period of time from 3 min to 24 hrs to obtain different C_0t values. Before annealing, the samples were heated for 10 min in a boiling water bath. After annealing, the samples were rapidly cooled, diluted 30 times with S_1 nuclease buffer (50 mM Na acetate, pH 4.5, 5 mM $ZnCl_2$, 0.28 M NaCl), and separated into three samples. Two of them were incubated with 10 units of S_1 nuclease for 1 hr at 45°C. Then all the three were put separately on GF/C glass filters, washed with 5% TCA, and counted. In each case, the blank S_1 resistance of cDNA (in the absence of annealing) was determined. It comprised usually 5% and was subtracted from the figure of hybridization. Also at high C_0t values, self-annealing of cDNA without a driver (which reached sometimes 3-5%) was considered.

RESULTS

1. Preparation of 5'-cDNA

The first step in preparing 5'-cDNA was the isolation of triphosphorylated 5'-end fragments with the aid of chromatography on hydroxyapatite. The modified elution procedure allows

one to achieve a better separation of the second peak containing these fragments and to obtain more reproducible separation (Fig. 1). Usually two cycles of chromatography were enough for adequate fractionation. At this stage, the second peak (B) contained about 0.5-1% of total RNA.

Analysis of the material of the second peak in DEAE chromatography after alkaline digestion revealed high enrichment with the alkaline resistant material possessing negative charge of -5 to -6. Its extent reached about 1.2% of the total

Fig. 1. Chromatography of pre-mRNA fragmented to 4-5S size on hydroxyapatite.

(a) First chromatography.

(b) Rechromatography of zone B from (a).

The arrows indicate the molarity of the elution buffer (NaPO_4 ; pH 6.9).

radioactivity. Appropriate calculations showed that practically each fragment contained such a group at the 5'-end. In contrast to equivalent material isolated from total pre-mRNA, the 5'-ends from peak B were much more sensitive to phosphatase treatment.

About 80-85% of them are transformed into nucleosides while only 15% are converted in the case of the -5 to -6 material from total pre-mRNA. Thus, separation with the modified method is the same as with the original one, but the technique is more reproducible and allows one to use higher amounts of RNA for fractionation.

The second step of the procedure is the removal of 3'-phosphate followed by polyadenylation. Treatment of alkaline phosphatase with diethylpyrocarbonate in the presence of Zn^{2+} as well as the high purity of riboadenyltransferase made it possible to conserve the size of RNA fragments equal to 80-120 nucleotides (4-5S) during these procedures. No RNA degradation took place. We checked the efficiency of polyadenylation in our experiments looking for binding of RNA to an oligo(dT) cellulose column. After polyadenylation from 50 to 80% of pre-mRNA fragments was able to bind to oligo(dT) cellulose. Thus, the major part of fragments could be transcribed.

The final step was the reverse transcription of pre-mRNA fragments. One can see from Fig. 2 that their size (4-5S) is about the same as that of RNA fragments used as a template. Thus, the full length of RNA fragments was transcribed.

2. Triphosphorylated 5'-ends of pre-mRNA consist of unique and moderately repetitive sequences

In the previous report from this laboratory, the kinetics of hybridization of 5'-pre-mRNA fragments to total cellular DNA was determined /6/. However, the upper limit of this RNA binding was not higher than 40-50% of the input, which does not allow one to make final conclusions. In the case of cDNA, the degree of the hybridization under the saturation conditions is about 70-80%. One can see (Fig. 3) that 5'-cDNA contains very small amounts of the material renaturing at $C_0t \leq 1$ moles $\times$ sec $\times$ l^{-1} . About a quarter of 5'-cDNA hybridizes in the region of moderately reiterated DNA sequences. The main

Fig. 2. Electrophoresis in 12% polyacrylamide gel of DNA complementary to the 5'-end fragments of pre-mRNA.

part of 5'-cDNA reassociated with unique sequences at $C_0t \geq 500$.

3. Triphosphorylated 5'-end fragments represent a subset of sequences in pre-mRNA

Preparation of 5'-cDNA allows one to determine the com-

Fig. 3. Hybridization of the 5'-cDNA with total DNA.

plexity of sequences adjacent to the triphosphorylated 5'-ends of pre-mRNA. 5'-cDNA was hybridized with the fraction of triphosphorylated 5'-end fragments (B) from hydroxyapatite chromatography (Fig. 4), and with total nuclear pre-mRNA. The $R_0 t_{1/2}$ of the reaction with 5'-pre-mRNA is about 0.4 moles $\times$ sec $\times$ l $^{-1}$, with total pre-mRNA is about 100 moles $\times$ sec $\times$ l $^{-1}$.

The complexity of 5'-end sequences can be calculated according to the equation $N = 5 \times 10^5 C_0 t_{1/2}$, followed from the data of Britten and Kohn [14]. It is equal to about 200 Kb, that corresponds to about 2×10^3 different sequences.

Thus, the complexity of the 5'-end fraction of pre-mRNA is about 250 times as low as that of total pre-mRNA. This result is in agreement with the fact that peak B contains about 0.5% or 1/200 of total pre-mRNA. However, the two figures cannot be compared directly as 5'-cDNA hybridization was driven by total pre-mRNA while the yield of 5'-RNA was calculated for nascent RNA enriched in triphosphorylated 5'-end fragments.

Fig. 4. Hybridization of the 5'-cDNA with 5'-end fragments of pre-mRNA obtained by hydroxyapatite chromatography (—○—) and with total nuclear pre-mRNA (—●—).

Therefore, it cannot be excluded that the sequences present in 5'-cDNA also occur in some other fragments, in particular in the capped ones.

4. Hybridization to mRNA

What we particularly were anxious to know in this work was whether sequences adjacent to triphosphorylated 5'-end are conserved in mature mRNA.

For this, the homology between cDNA and polysomal mRNA was measured in direct hybridization experiments. The results obtained with different samples of cDNA were somewhat variable. To quantitate the data, the same cDNA samples were also hybridized to an excess of nuclear pre-mRNA under the saturation conditions. In this case, the maximal binding varied for different preparations from 60-70 to 80-90%, possibly depending of such uncontrolled minor differences between samples as the percentage of short chains, etc. Since cDNA had been transcribed from pre-mRNA or its fractions, these figures were considered as a limit of binding (100%) and the figures obtained in experiments where mRNA was tested were corrected considering this hybridization efficiency. After correction, the results obtained with different cDNA samples were essentially the same.

One can see from Fig. 5 that about 18% of total cDNA hybridized to mRNA. After correction for the reaction efficiency, the 25% binding was obtained. Thus, from one-fifth to one-fourth of the total pre-mRNA sequences are represented in polysomal mRNA.

Even higher hybridization to mRNA was observed in the case of 5'-cDNA. Up to 35% of 5'-cDNA becomes resistant to nuclease S_1 after annealing to mRNA at $R_0 t$ equal to 20-100 moles $\times$ sec $\times$ l^{-1} . After correction for the reaction efficiency, 45-50% of the binding was obtained in different experiments (Fig. 6). The hybridization takes place at low $R_0 t$ values. Thus, it cannot be explained by the presence of nuclear RNA contaminations in mRNA samples.

One may object that even mild alkaline treatment during RNA shearing can make imidazole rings open in some part of caps leading to the loss of one positive charge. Such modified

Fig. 5. Hybridization of the DNA complementary to the total nuclear pre-mRNA with polysomal mRNA. (Corrected hybridization % is calculated on the basis of efficiency of hybridization reaction).

capped fragments might contaminate the fraction of triphosphorylated 5'-end fragments.

Therefore, in control experiments, pre-mRNA for fraction-

Fig. 6. Hybridization of the 5'-cDNA with polysomal mRNA.

(—○— pre-mRNA for isolation of the 5'-end fragments was fragmented by alkali; —▲— by pancreatic RNAase).

nation and reverse transcription was sheared by mild treatment with pancreatic RNAase. The hybridization of 5'-cDNA to mRNA in this case was the same as described above. As RNAase does not interfere with caps, the afore mentioned explanation of the results seems unlikely. Furthermore, the possibility of hybridization of 5'-cDNA with some snRNA associated with mRNA was checked. The existence of these snRNA was demonstrated earlier /15/. To this end, mRNA was denatured in formamide and its heavy fraction was purified by gel filtration through a Sephadex G-200 column under the denaturing conditions. The high level of 5'-cDNA hybridization to this mRNA sample was obtained. Thus, we may conclude that sequences hybridizing with 5'-cDNA are present in mRNA itself. Apparently, a significant fraction of the starting fragments of primary transcripts is conserved and reaches polysomes as a part of mRNA chains.

5. Some other properties of triphosphorylated 5'-end fragments in pre-mRNA

In a previous paper /6/, the enrichment of triphosphorylated 5'-ends in oligo(U) sequences was shown by hybridization to poly(A) Sepharose. This result has been now confirmed by chromatography of 5'-cDNA on oligo(dT) cellulose. As can be seen from Table 1, 15% of 5'-cDNA as compared to only 5% of total cDNA are bound to the column. Thus, many leading sequences in pre-mRNA contain oligo(U).

It was also shown previously that 5'-end fragments lacked hairpin-like structures. Now we have checked whether they contain sequences most often occurring in the ds regions of pre-mRNA, namely B1 and B2 sequences cloned recently in our laboratory /16/. 5'-cDNA was hybridized to an excess of DNA prepared from clones containing B1 and B2 sequences. Only tracer amounts (less than 0.2%) of 5'-cDNA were bound to filters (data not shown). Thus, no enrichment in these sequences takes place for 5'-cDNA.

Table 1. Chromatography of the 5'-cDNA and total cDNA (complementary to the total nuclear RNA) on oligo(dT) cellulose column

cDNA analyzed	cDNA eluted by 0.4 M NaCl, cpm	cDNA eluted by H ₂ O at 40°C, cpm	fraction of cDNA containing oligo(dA) sequences
5'-cDNA	1900	320	14.4%
total cDNA	6020	330	5.2%

DISCUSSION

Although nuclear DNA-like RNA (dRNA, hnRNA) was known since 1962 /10,17,18/ and the pre-mRNA concept was first formulated at about the same time /19,20/, the idea that at least the major part of this RNA is a high molecular weight precursor of mRNA has been finally proved only recently. One of the experiments supporting the hypothesis is that of Hames and Perry /21/ who isolated DNA complementary to mRNA (mDNA) and hybridized with it rapidly labeled nuclear pre-mRNA of different sizes. From 16 to 25% of the latter was found in the hybrid showing a high degree of homology between mRNA and pre-mRNA. In the present paper, a different approach to this problem has been used, namely the hybridization of cDNA transcribed from total nuclear pre-mRNA or its fraction to polysonal mRNA. To make the reverse transcription possible, the fragmented pre-mRNA was polyadenylated. This method was used at first time by A. Hell et al. for reverse transcription of rRNA /22/.

The use of cDNA complementary to triphosphorylated fragments has several advantages over previous procedures. The high specific activity of cDNA allows one to perform the reaction in saturation conditions. The complementarity of cDNA to pre-mRNA makes it possible to check the homology to mRNA in direct hybridization experiments avoiding more complicated competition experiments.

The main result obtained in this paper is the demonstration that about half of triphosphorylated 5'-end fragments of

pre-mRNA are homologous to polysomal mRNA. This suggests the conservation of about half of the starting fragments of pre-mRNA in the course of pre-mRNA processing and mRNA maturation.

The results obtained are in certain contradiction with the previous data on the absence of competition between mRNA and pre-mRNA fragments containing pppN-groups in hybridization to DNA /23/. However, the early results were obtained under the conditions of low C_0t hybridization where only repetitive 5'-fragments could be bound. In the present paper, we found that less than 30% of triphosphorylated 5'-ends in pre-mRNA were represented by copies from intermediate repeats, the other being copies from unique sequences.

There is sufficient evidences to the conclusion that mRNA sequences are located at the 3'-ends of pre-mRNA /23-26/. The distance between the 5'-triphosphate group and the polyadenylated 3'-end in pre-mRNA is much greater than the size of mature mRNA /6/. If half of triphosphorylated 5'-end sequences are also conserved in mature mRNA, one is bound to suggest that the splicing step is involved in the processing of a significant fraction of pre-mRNA. Thus, the splicing seems to be a widely spread event in processing of mammalian pre-mRNA. Of course, other experiments based on different approaches are necessary for final conclusion to be reached.

Not all triphosphorylated 5'-end fragments are homologous to mRNA sequences. They may be present in pre-mRNA with another processing program including, for example, the elimination and decay of the 5'-end sequences of original transcripts. Such a type of processing was demonstrated for pre-rRNA /27/. Further studies on triphosphorylated 5'-end sequences in total pre-mRNA and particularly in individual pre-mRNAs using the techniques developed in the present paper might provide new information on the mechanism of pre-mRNA processing in eukaryotic cells.

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