Volume 15 Number 24 1987 Nucleic Acids Research

Nucleotide sequence and characterization of the transcript of a *Dictyostelium* ribosomal protein gene

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Received September 16, 1987; Accepted November 10, 1987

ABSTRACT

Dictyostelium ribosomal protein mRNAs are subject to developmental regulation of both their translation and their stability. In order to consider whether such post-transcriptional regulation can be attributed to structural features of the mRNAs, we have cloned and sequenced a 1.9 kb EcoRI genomic DNA fragment which contains the gene for the *Dictyostelium* ribosomal protein 1024 (rp1024). The rp1024 gene contains a single intron of 350 bp which begins just after the fourth codon of protein coding sequence. Transcription begins 11 to 28 bp upstream from the initiator ATG in a pyrimidine rich region which is preceded by an oligo(dT)$_{10}$ stretch, but which lacks a TATA box in the expected position. Processing of the 3'end occurs at either of two sites, resulting in two types of transcript which are present in equimolar amounts in both vegetatively growing and developing cells. Therefore, their relative abundance shows no correlation with the changes in translatability and stability of r-protein mRNAs which occur during development. A comparison of the sequence of the 5'-untranslated region of rp1024 mRNA to those of other *Dictyostelium* mRNAs shows that it differs significantly, primarily in its relatively high G+C content.

INTRODUCTION

In both eucaryotes and procaryotes, ribosomal proteins accumulate coordinately in response to a variety of changes in cellular physiology. An important element in the regulation of ribosome biosynthesis in procaryotes, *E. coli* in particular, is a well-characterized system of feedback control of r-protein mRNA translation (1). There is also evidence in a wide range of eucaryotes that translational regulation can play a role in controlling r-protein synthesis although the molecular mechanism is unknown. Controls have been demonstrated at several other levels including synthesis, stability, and processing of the ribosomal components (2,3).

To facilitate our studies of ribosome biogenesis in the eucaryote *Dictyostelium discoideum*, we have previously constructed and characterized cDNA plasmids complementary to six different r-protein mRNAs (4,5). We have shown that during development in *Dictyostelium*, r-protein gene expression is regulated by at least two separate post-transcriptional events involving

changes first in the translational activity and then later in the stability of the r-protein mRNAs (5). When amoebae are starved and induced to undergo development, r-protein synthesis is decreased by a specific reduction in the amount of r-protein mRNA associated with polysomes. Although the r-protein mRNAs are translated at very low levels, they remain present in developing cells in amounts comparable to those found in vegetatively growing cells. After about 10 hours of development, when the cells are forming tight cell-cell contacts, there is a rapid decrease in r-protein mRNA levels (5).

In order to begin to examine structural elements of the r-protein mRNAs which may be involved in the regulation of their stability and rate of translation, we have now cloned and sequenced a genomic fragment which encodes the Dictyostelium r-protein designated rp1024. The sequence contains several features which have been shown to be characteristic of Dictyostelium genes, and some which have been found in r-protein genes of other eucaryotes.

METHODS

General procedures

Growth and development of D. discoideum (strain Ax-3), isolation of DNA, RNA, and poly(A)$^+$ RNA were as described previously (5,6). Plasmid DNA preparation, Southern and northern blotting, DNA labeling, and cloning procedures all followed standard methods (5,7). Methods for hybrid selection of specific mRNAs and two-dimensional electrophoresis of ribosomal proteins have been described in detail (4).

Construction and selection of a genomic plasmid prp1024g-A which contains the gene for r-protein 1024

Southern blots of Dictyostelium nuclear DNA probed with labeled insert DNA from the cDNA plasmid prp1024 (5, this paper) showed that the r-protein 1024 is encoded by a single copy gene and is contained on an EcoRI fragment of ~1.9 kb (data not shown). A limited genomic plasmid library was constructed by the insertion of EcoRI fragments ranging in size from 1.8 kb to 2.0 kb into the EcoRI site of the vector pUC8 and subsequent transformation into E. coli strain TB1 (Δlac-pro, str A, ara, thi, ø80 dlacZ ΔM15, hsdR). Colonies were screened by hybridization to insert DNA from prp1024 labeled by nick-translation and five isolates were selected, each of which contained an apparently identical 1.9 kb EcoRI genomic fragment. One isolate was retained for further analysis and designated prp1024g-A.

The 1.9 kb genomic DNA insert of prp1024g-A was labeled and used to probe northern blots of RNA isolated at 2 hour intervals throughout development. The pattern of accumulation was identical to that seen using the cDNA plasmid

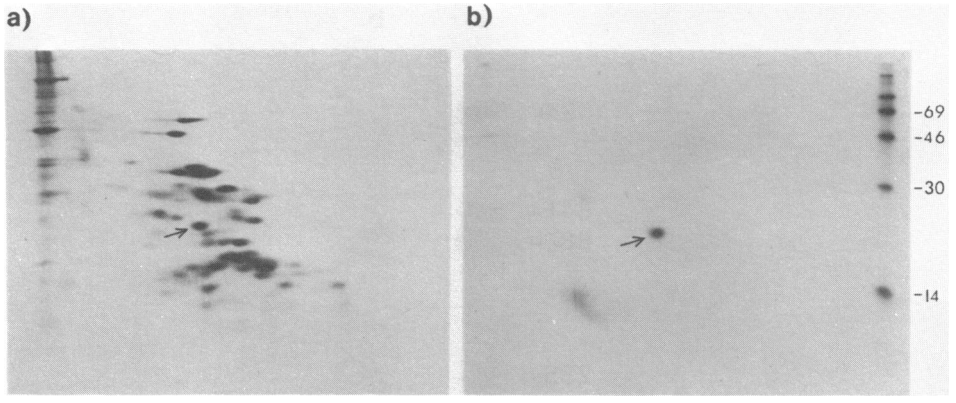


Figure 1. Two dimensional electrophoresis of the in vitro translation products of mRNA selected by hybridization to prp1024 DNA. Panel a: A stained gel of electrophoretically separated Dictyostelium r-proteins. The spot which co-migrates with the translation product shown in panel b is indicated by an arrow. Panel b: Total RNA from vegetatively growing cells was hybridized to prp1024 DNA immobilized on nitrocellulose. Specifically bound RNA was eluted and translated in rabbit reticulocyte lysates using ^{35}S-methionine. The products were mixed with unlabeled marker r-proteins and electrophoresed in two dimensions. ^{14}C-labeled size marker proteins were included in the second dimension. The gel was stained with Coomassie Brilliant Blue, treated with Autofluor (National Diagnostics), dried, and exposed for autoradiography.

prp1024 as probe (5, data not shown). No additional mRNAs were detected, indicating that this genomic fragment does not encode substantial portions of any other mRNAs or transcribed repeats.

Sequencing strategy

The genomic plasmid, prp1024g-A, was linearized at the BamHI site (in the pUC8 polylinker) and digested with Bal 31 at a concentration of 0.5 mg/ml DNA and 10 units/ml Bal 31 nuclease for 2.5, 5, 15, and 30 minutes at 30°C in order to generate a series of deletions of increasing length. The deleted ends were made blunt by filling in with dNTPs and DNA polymerase I, Klenow fragment, and then released from the pUC8 vector by digestion with EcoRI. The shortened insert fragments were further size fractionated by electrophoresis in low melting temperature agarose and subcloned into EcoRI and HincII digested M13, mp10 for sequencing.

To obtain a plasmid which could be linearized and deleted at the other end of the insert, DNA from the M13 subclone with the smallest deletion (but devoid of the EcoRI site at the deleted end) was digested with PstI and EcoRI and the released insert was ligated into PstI and EcoRI digested pUC8. This construction is prp1024g-B. A series of Bal 31 deletions was generated from

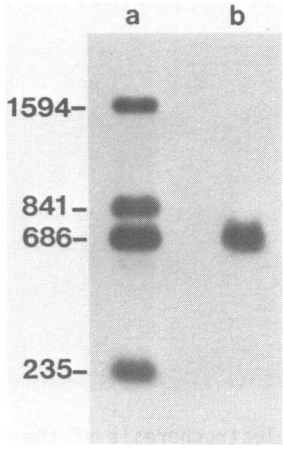


Figure 2. Size determination of rp1024 mRNA by northern analysis. RNA was treated with glyoxal, electrophoresed in a 1 percent agarose gel buffered with 10 mM sodium phosphate, pH 7.0, and transferred by blotting to Zeta-Probe (Bio-Rad). The filter was probed by hybridization to prp1024 insert DNA labeled by nick-translation. Lane a: Markers of the indicated sizes (in nucleotides) generated by in vitro transcription with α^{32}P-UTP from recombinant pSP65 plasmids. Lane b: Total RNA from vegetatively growing cells.

the EcoRI site, filled in, released with PstI, and subcloned into PstI and SmaI digested M13, mp11. Sequencing was done by the dideoxy strand termination method (8).

S1 protection assays

Probes for S1 protection analysis (9) of the 5' and 3' ends of transcription were prepared from prp1024g-B digested with AccI, which cuts at two positions within the coding region (see Figure 5). The 3' ends were labeled by reaction with α-^{32}P-dCTP and DNA polymerase I, Klenow fragment. Alternatively, the 5' ends were labeled after treatment with calf intestine alkaline phosphatase by reaction with γ-^{32}P-ATP and T4 polynucleotide kinase. The 3' end-labeled DNA was further digested with EcoRI and the 599 bp fragment was isolated by electrophoresis in low melting temperature agarose and purified by extraction with CETAB/butanol (10). Similarly, a 1103 bp fragment was isolated from the 5' end-labeled DNA after further digestion with PstI.

The 3' end-labeled AccI/EcoRI fragment and the 5' end-labeled AccI/PstI fragment were hybridized separately to total RNA from vegetatively growing cells in 80 percent formamide, 40mM Pipes, pH 6.5, 1mM EDTA, 0.4 M NaCl at

45°C (3' end) or 43°C (5' end) for 3.5 hours (11,12). Hybrids were digested with S1 nuclease at 0, 30, or 135 units per 100 ul reaction for 1 hour at 37°C. For the analysis of the 3' ends of transcripts from developing cells, S1 nuclease was used at 100 units per 100 ul reaction. Protected fragments were sized by electrophoresis in 5 percent acrylamide-urea sequencing gels (13).

Primer extension

A 63 bp HinfI fragment from prp1024g-B, complementary to rp1024 mRNA in a region beginning 46 bp downstream from the splice junction, was isolated and ^{32}P-labeled at its 5' ends. The fragment was denatured and re-annealed with poly(A)$^+$ RNA under conditions described above for S1 nuclease mapping. RNA/DNA hybrids were separated from the non-complementary primer fragment strand by oligo(dT)-cellulose chromatography (14). The hybridized primer was extended with AMV reverse transcriptase in the presence, or absence, of dideoxynucleoside triphosphates as described (15), and the extended products were analyzed by electrophoresis in 5 percent acrylamide-urea sequencing gels.

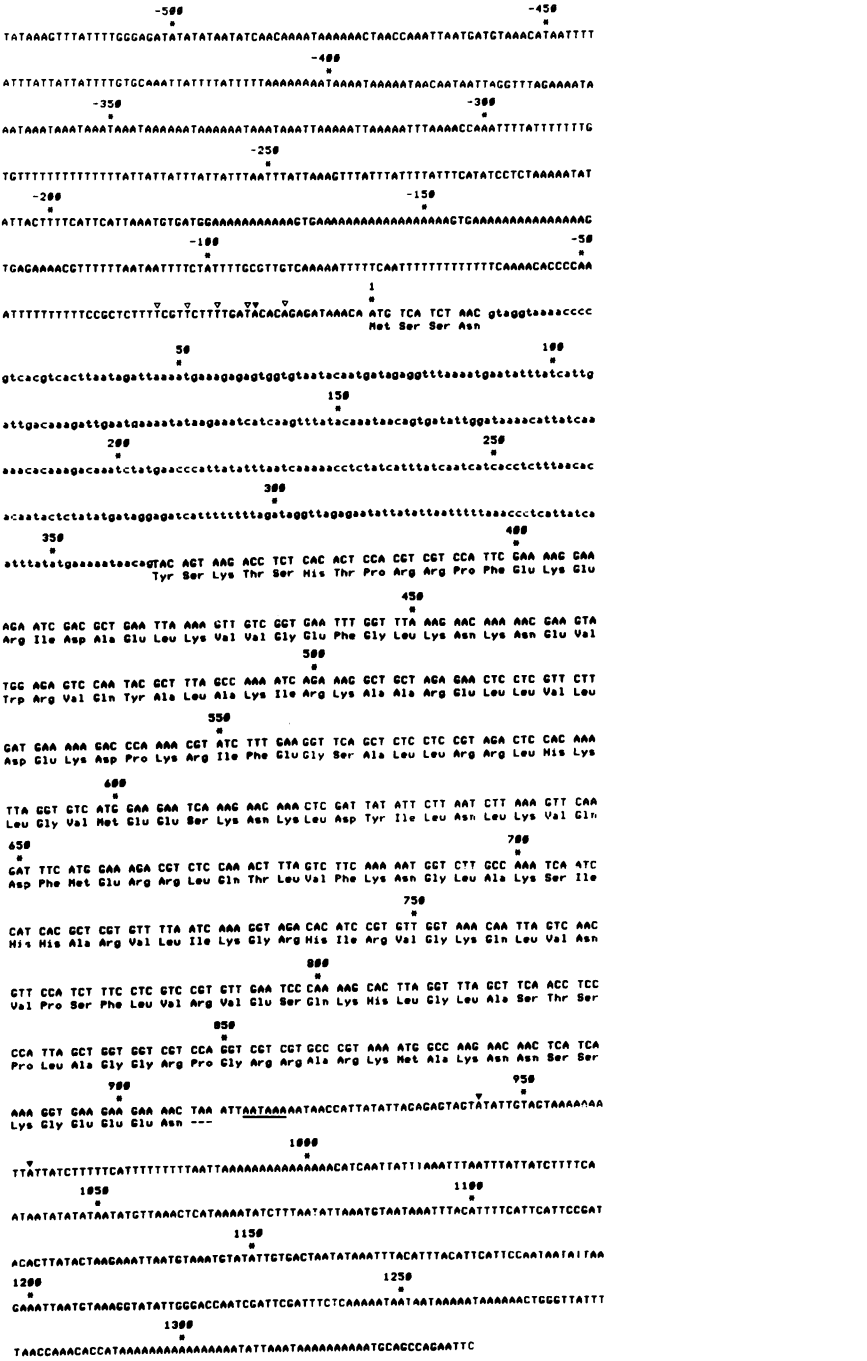
RESULTS AND DISCUSSION

The cDNA plasmid prp1024 encodes a ribosomal protein

The plasmid prp1024 contains a cDNA insert in the PstI site of pUC8 and was originally characterized as encoding a stable mRNA (5). Using techniques similar to those described for five other plasmids which encode Dictyostelium r-proteins (4), we have shown that prp1024 is also complementary to an r-protein mRNA. Figure 1 shows that *in vitro* translation of mRNA selected by hybridization to immobilized prp1024 DNA yields a product that comigrates during two dimensional gel electrophoresis with a ribosomal protein of MW = 21,000. This r-protein (rp1024) is encoded by an mRNA ~680 nucleotides long, as shown by northern blot analysis of total RNA from vegetatively growing cells using prp1024 as probe (Figure 2).

Molecular cloning and sequencing of the genomic region encoding r-protein 1024

A 1.9 kb EcoRI genomic DNA fragment encoding r-protein 1024 was cloned in the vector pUC8 and selected using prp1024 as described in Methods. The sequence of this genomic region, shown in Figure 3, was determined using the dideoxynucleotide strand termination method (8). In order to obtain accurate sequence information, even in the highly A+T rich non-coding regions of this gene, we found it helpful to use a closely spaced set of Bal 31 deletions generated from either end of the gene in the plasmids prp1024g-A and prp1024g-B and subcloned into M13 vectors mp10 and mp11, respectively (see



Methods). The sequence was determined from multiple, overlapping subclones, not all of which are shown in the strategy depicted in Figure 4.

Characterization of the transcribed region of the rp1024 gene

The sense and anti-sense strands were identified by hybridization of labeled DNA prepared from single-stranded M13 constructs to northern blots of RNA from growing cells (data not shown). The presence of a long open reading frame in the sense strand as well as the extremely high A + T content of Dictyostelium DNA in non-coding regions (16) allowed us to identify roughly the transcribed region of the gene. The extent of transcription was mapped more precisely by S1 nuclease protection and primer extension.

In order to locate the 5' end of transcription, S1 protection assays were done utilizing the AccI-PstI fragment of prp1024g-8, 5' end-labeled at the AccI site (see Figure 5). However, the size of the fragment protected by hybridization to total RNA identified a 5' end with characteristics which suggested that it was an intron splice site and not the 5' start of transcription. Protected sequence was immediately preceded by the dinucleotide AG and there was no methionine codon followed by an open reading frame sufficiently long to encode rp1024. Comparison to sequence obtained from the cDNA plasmid prp1024 confirmed that this was an intron 3' acceptor site and located the 5' donor site 350 bp upstream. The portion of the genomic region which is included in the cDNA plasmid is shown in Figure 4. The single 350 bp intron in the rp1024 gene has an A+T content of 75 percent, making it both longer and less A/T rich than all other Dictyostelium introns so far characterized. Previously identified introns from Dictyostelium have an average length of ~100 nucleotides and an A+T content of >90 percent. The sequence at the splice junctions, /GTAGGT....AG/, is in agreement with both Dictyostelium and the general consensus (16,17).

The 5' start of transcription was mapped by primer extension. A 63 bp HinfI fragment complementary to the mRNA in a region starting 46 bases downstream of the intron junction was used as a primer and extended by reverse transcription in the presence of dideoxynucleoside triphosphates. The

Figure 3. DNA and derived amino acid sequence of the gene encoding r-protein 1024. The DNA sequence of a 1.9 kb EcoRI fragment containing the r-protein 1024 gene was determined using the dideoxynucleotide strand termination method. The major 5' transcriptional start site is indicated by a closed triangle and minor start sites are indicated by open triangles. The 350 bp intron is depicted in lower case letters. Two alternative 3' transcript ends are indicated by closed triangles and the AATAAA polyadenylation signal is underlined.

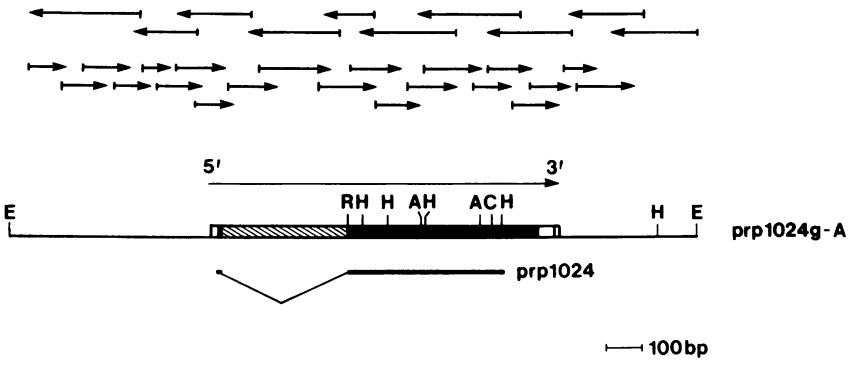


Figure 4. Sequencing strategy and gene structure of the region which encodes r-protein 1024. A partial restriction map and the structural organization of the 1.9 kb EcoRI fragment contained in the genomic clone, prp1024g-A, is shown in the lower part of the figure. 5'- and 3'-untranslated regions are depicted by open bars, the intron by a hatched bar, and coding region by solid bars. The extent and direction of transcription is indicated by the arrow above the map and the region contained in the cDNA clone, prp1024, is indicated below the map. The arrows in the upper part of the figure indicate the extent and direction of sequence analysis from a set of deletions generated by Bal 31 digestion as described in Methods. (E = EcoRI, R = RsaI, H = HinfI, A = AccI, C = HincII.)

sequence determined from the 5' end of the mRNA confirmed the intron splice junction and located the 5' ends of the transcripts. Figure 6 shows that there is some heterogeneity at the 5' end with a major transcriptional start site at -15 and others at -11, -16, -20, -24, and -28 relative to the ATG start of translation. Transcription begins in a pyrimidine rich region which is unusual for Dictyostelium start sites which are more often A/T rich. However, this region is strikingly similar to the transcriptional start sites of the r-protein genes of higher eucaryotes (14,18,19,20). The oligo(dT)$_{10}$ tract just upstream of this region is highly characteristic of Dictyostelium where all known transcripton start sites except one are preceded by a stretch of dT residues (16). The exception is the gene encoding UDP glucose pyrophosphorylase where the oligo(dT) tract is located in the anti-sense strand, rather than the sense strand (21).

In other Dictyostelium genes studied, a TATA box with the consensus sequence TATAAA($^{T}_{A}$)A has been found just upstream of the oligo(dT) tract (16). There is no homology with that consensus in that position in the rp1024 gene, although the sequence AATAATTT occurs 96 bp upstream of the major transcriptional start site. This sequence is homologous at 5 out of 8 positions to the Dictyostelium TATA box consensus, but we have no data

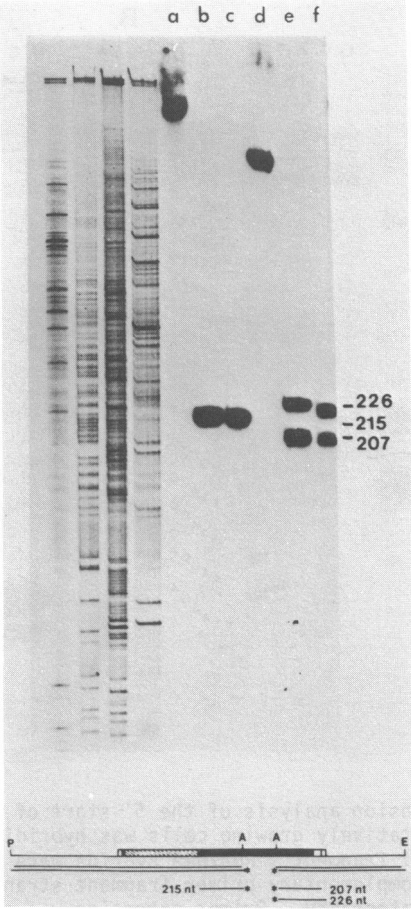


Figure 5. S1 protection analysis of the extent of transcription. The plasmid prp1024g-B was used to generate both a PstI/AccI fragment, 5' end-labeled at the AccI site, and an EcoRI/AccI fragment, 3' end-labeled at the Acc I site. These fragments were hybridized, in separate reactions, to total RNA isolated from vegetatively growing cells. Hybrids were digested with S1 nuclease and protected fragments were sized by electrophoresis in 5 percent acrylamide-urea gels, using sequence ladders from an unrelated sequence as markers. Lanes a-c show protected 5' end-labeled fragments after reaction with 0, 30, or 135 units of S1 nuclease per 100 ul reaction, respectively. Lanes d-f show protected 3' end-labeled fragments after reaction with 0, 30, or 135 units of S1 nuclease per 100 ul reaction, respectively. Fragment sizes are indicated in nucleotides. (P = PstI, A = AccI, E = EcoRI.)

regarding its functional role. Several r-protein gene sequences from other eucaryotes have been reported to lack TATA boxes (14,19,20).

S1 nuclease mapping of the 3' end of transcription showed that there are

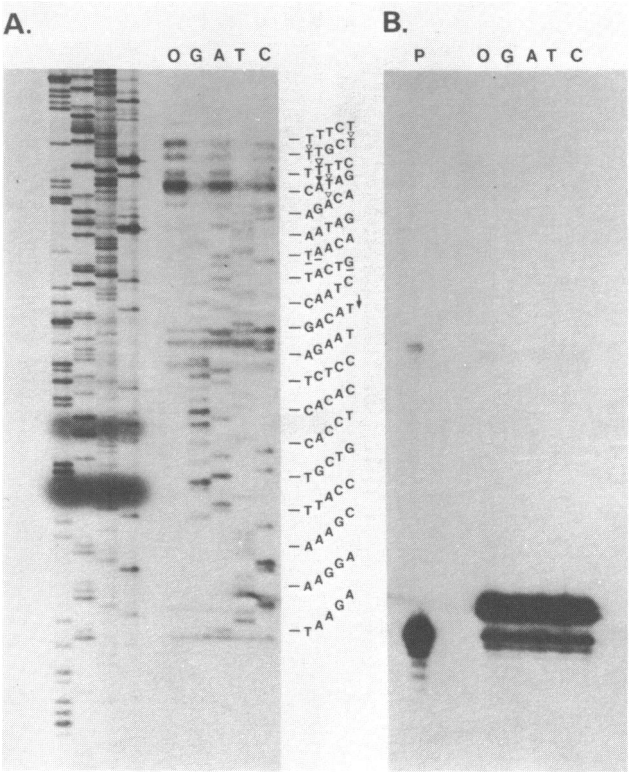


Figure 6. Primer extension analysis of the 5' start of transcription. Poly(A)$^+$ RNA from vegetatively growing cells was hybridized with a 5' end-labeled 63 bp HinfI fragment. RNA/DNA hybrids were separated from unhybridized and non-complementary primer fragment strands by oligo(dT)-cellulose column chromatography. Primer extension was carried out with reverse transcriptase in the presence of dideoxynucleoside triphosphates using as template hybrids collected from either the bound fraction (panel A) or the unbound fraction (panel B). Lanes labeled O, G, A, T, and C show the products of reactions with no ddNTP or added ddGTP, ddATP, ddTTP, and ddCTP, respectively. The lane labeled P contains unreacted primer fragment and sequence ladders from unrelated sequence are included as size markers. The sequence derived from the 5' end of the mRNA is indicated as the sense strand to facilitate comparison to the sequence presented in Figure 1. The arrow indicates the position of the intron splice junction, the initiator ATG is underlined, and positions identified as 5' transcription start sites are indicated as in Figure 1.

two different sized transcripts present in approximately equimolar amounts in the RNA of vegetatively growing cells (Figure 5). Hybridization of total RNA with the AccI-EcoRI fragment, labeled at its 3' end at the AccI site, resulted in protection of two fragments 207 or 226 nucleotides long. 3' end processing

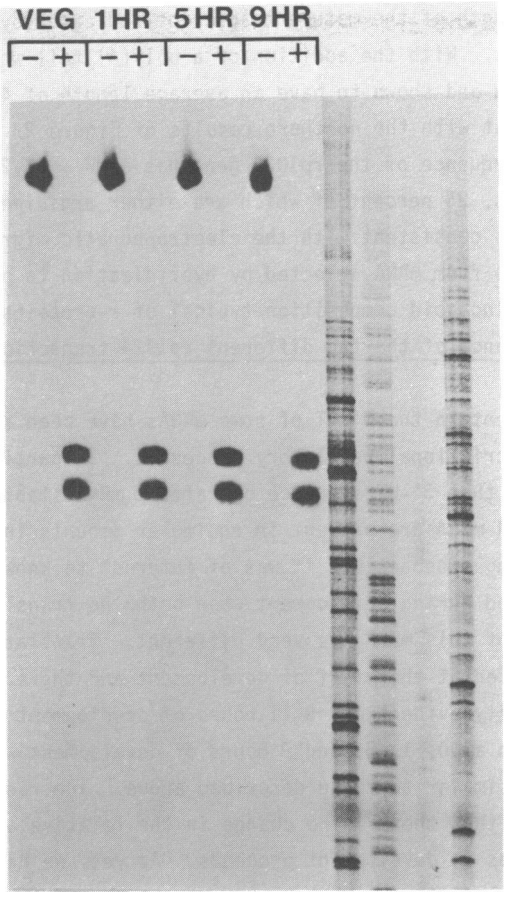


Figure 7. S1 protection analysis of the 3' ends of r-protein 1024 mRNA present in developing cells. Total RNA was isolated from cells after 0 hour (vegetatively growing), 1 hour, 5 hours, and 9 hours of development on buffer saturated filter pads. The RNA was hybridized with the prp1024g-B EcoRI/AccI fragment, 3' end-labeled at the AccI site, as shown in Figure 3. Hybrids were digested with 100 units S1 nuclease per 100 ul reaction (lanes marked "+") or left undigested (lanes marked "-") and the protected fragments were sized by 5 percent acrylamide-urea gel electrophoresis. Unrelated sequence ladders are present as size markers.

and polyadenylation apparently occurs at either of two alternative sites, resulting in transcripts with 3'-untranslated regions (3'-UTs) of 35 or 54 nucleotides. There is a single AATAAA sequence which begins 32 bp or 51 bp upstream of the polyadenylation sites, and in the A+T rich 3'-UT there are at least two other sequences with partial homology to that consensus polyadenylation signal.

The overall length of the mature transcript indicated by sequence analysis is ~630 nucleotides. With the addition of a poly(A) tail which has been previously measured and shown to have an average length of 40-60 A's (5), this is in good agreement with the northern results of Figure 2. The protein derived from the sequence of the rp1024 gene has a MW = 21,205, a pI = 10.5, and 185 amino acids, 25 percent of which are either arginine, lysine, or histidine. This is consistent with the electrophoretic migration of rp1024 translated in vitro from mRNA selected by hybridization to prp1024 DNA (Figure 1) and with the amino acid composition typical of r-proteins in general.

The relative abundance of the two different rp1024 transcripts during development

Sequences present in the 3'-UT of some mRNAs have been shown to play a role in post-transcriptional regulatory processes. In particular, several studies have shown that 3'-UT sequence can affect mRNA stability (22,23). The two sizes of rp1024 mRNA are present in equimolar amounts in total RNA of vegetatively growing amoebae, but it was of interest to know if this distribution changed during development when both the translational activity and the stability of this mRNA are very different. Translational activity is substantially reduced at the start of development and there is an apparent decrease in stability evident by ~9-11 hours of development (5). Total RNA isolated from cells at 0, 1, 5, and 9 hours of development was used in S1 protection assays similar to those described above. The results, shown in Figure 7, indicate that there is no change in the relative abundance of the two transcript sizes as development proceeds. As yet, we have no data to suggest a functional difference between the two types of transcript.

The 5'-untranslated region of rp1024 mRNA

In many studies, determinants of translational efficiency have been shown to reside in the 5'-UT of mRNAs. These include cap structures (24), secondary structure (25), and sequence context surrounding the AUG (26). We have compiled sequence data from the region surrounding the initiator AUG of 14 Dictyostelium mRNAs in order to derive a Dictyostelium consensus for comparison to the 15 nucleotide 5'-UT of rp1024 mRNA as well as to the consensus found for other eucaryotes (see Table 1). In our compilation we have included only one member from each of the gene families encoding actin (actin-8), discoidin (discoidin 1α), and M3 (M3L) in order to avoid any bias, particularly towards actin mRNA. However, inclusion of the 14 additional actin mRNA sequences, the 2 remaining discoidin mRNAs, and M3R mRNA does not significantly change the consensus.

It has been noted previously that the 5'-UTs of Dictyostelium mRNAs are highly A and U rich (16) and all initiating AUG codons are preceded by at least one (16) and as many as 24 A residues (35). This is reflected in the consensus which shows a preference for A at every position as far as -15 from the AUG. The second most prevalent nucleotide is U, while G and C are rare. There is also a strong preference (71 percent) for A at position +6.

The 5'-UT of rp1024 mRNA differs from the consensus in that it contains a relatively high percentage of G and C residues (>30 percent). Only two other Dictyostelium mRNAs have comparably G+C rich 5'-UTs. EB4 mRNA has a 5'-UT of >300 nucleotides and is ~30 percent G+C (30) although the identification of the initator AUG may be questioned. Contact site A mRNA has a 5'-UT of at least 120 nucleotides (sequenced from a cDNA clone) and is ~28 percent G+C (33). Both of these mRNAs differ from rp1024 mRNA in the unusual length of their 5'-UTs and in that they contain multiple upstream AUG triplets.

Rp1024 mRNA also shows a greater than usual variation from the consensus immediately surrounding the initiator AUG. While it does contain the as yet invariant A at position -1, and the consensus A at -3, it is the only Dictyostelium mRNA with a C at any position from -1 to -4. In this regard, rp1024 mRNA more closely conforms to the consensus derived from other eucaryotic translation start sites (see Table 1). It will be interesting to learn whether these differences are shared by other r-protein mRNAs and whether they help to account for the low translational activity of these mRNAs during development.

ACKNOWLEDGEMENT

This work was supported by the Public Health Service grant GM27757 from the National Institutes of Health.

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