Proc. Nat. Acad. Sci. USA
Vol. 71, No. 5, pp. 1607–1611, May 1974

Transcription of Polydeoxythymidylate Sequences in the Genome of the Cellular Slime Mold, *Dictyostelium discoideum*

[messenger RNA/poly(A)/depurination/hybridization]

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Communicated by James E. Darnell, Jr., December 21, 1973

ABSTRACT **Messenger RNA of the cellular slime mold, *Dictyostelium discoideum*, contains about equimolar amounts of two classes of poly(adenylic acid) [poly(A)]; one is about 25 nucleotides long and the second about 100 nucleotides long. At least half of the messenger RNA molecules contain one sequence each of poly(A)$_{25}$ and poly(A)$_{100}$; both poly(A) sequences are located near the 3′ end of messenger RNA, and the kinetics of their appearance on messenger RNA precursor indicates that poly(A)$_{25}$ is added before poly(A)$_{100}$. *Dictyostelium* nuclear DNA contains 14,000–15,000 sequences of poly(dT)$_{25}$ which could code for the smaller poly(A) residues. The poly(A)$_{100}$ must be added post-transcriptionally. The poly(dT)$_{25}$ sequences are interspersed throughout the genome and may well represent transcription termination regions.**

Messenger RNA of mammalian cells contains, at the 3′ end, a sequence of poly(adenylic acid) [poly(A)] of about 200 bases (1–9). Twenty to 40% of the giant heterogeneous nuclear RNA (HnRNA) molecules found in these cells also contain a poly(A) sequence at their 3′ end (6, 8). Because the poly(A) in the HnRNA is present at the 3′ end, it has been suggested that the region of the HnRNA molecule nearest the 3′-end poly(A) is the material precursor of mRNA (7, 9, 10).

There is evidence that these poly(A) sequences are not encoded in DNA, but are added post-transcriptionally: (*a*) cordycepin blocks synthesis of poly(A) and appearance of mRNA into the cytoplasm, but does not inhibit synthesis of HnRNA (5, 7, 8, 11, 12); (*b*) the number and size of poly(dT) sequences in mammalian nuclear DNA has been determined by isolation of polypyrimidine sequences in DNA after depurination; these poly(dT) sequences could account for only a very small fraction of the poly(A)$_{200}$ sequences in mRNA (13); and (*c*) adenovirus type-2 mRNA contains poly(A) sequences of 100–200 bases, yet adenovirus DNA contains no poly(dT) sequences of equivalent size detectable by RNA DNA hybridization (14).

We previously showed that mRNA from *Dictyostelium* contains a poly(A) sequence of 40–150 nucleotides, with a mean size of 100 at the 3′ end (15). We report here that mRNA also contains a sequence of poly(A) of length 25 bases. Further, we show that *Dictyostelium* DNA contains sequences of poly(dT) of sufficient size, number, and distribution to encode the poly(A)$_{25}$ sequences [but not the poly(A)$_{100}$] found in mRNA.

Abbreviation: HnRNA, heterogeneous nuclear RNA.

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MATERIALS AND METHODS

Materials. Both ^{3}H-labeled and unlabeled poly(U) and poly(A) were obtained from Miles Laboratories. Carrier-free [^{32}]phosphoric acid, [^{3}H]adenine (15 Ci/mmol), and [α^{32}P]-ATP (5 Ci/mmol) were purchased from New England Nuclear Corp. Cyanogen bromide-activated Sepharose 4B was a product of Pharmacia Fine Chemicals. RNases A and T1 were obtained from Worthington Biochemical Co. and Calbiochem.

Labeling of Dictyostelium RNA. Amoebae of *Dictyostelium discoideum* AX-3, growing in axenic medium (18, 19), were used. Conditions of labeling cells with $^{32}PO_4^{-3}$ and [^{3}H]adenine and isolation of mRNA and nuclear mRNA precursors have been detailed (15, 16). *Dictyostelium* nuclei were isolated and utilized for cell-free RNA synthesis as described (17).

Isolation of Poly(A). Either total RNA, or poly(A)-containing RNA purified by chromatography on poly(U)–Sepharose, was used. RNAs were digested at 37° for 30–60 min in 0.36 M Na^+ by 10 units/ml of RNase T_1 and 5 μg/ml of RNase A. Sodium dodecyl sulfate (0.5%) and proteinase K (1 mg/ml) were added to terminate the reaction. After an additional incubation at 30°, the mixture was extracted with phenol–chloroform and the poly(A) material was purified by adsorption to and elution from poly(U)–Sepharose or oligo(dT)–cellulose (15, 16). Finally the poly(A) was precipitated with ethanol, with *Escherichia coli* tRNA as carrier. The size and base composition of the poly(A) sequences were the same whether or not the poly(A)-containing RNA was first purified by chromatography on poly(U)–Sepharose.

Poly(U)- and Poly(A)–Sepharose. Poly(A)–Sepharose was prepared, from cyanogen bromide-activated Sepharose 4B, and used exactly as detailed for poly(U)–Sepharose (15, 16).

Analysis of Poly(A) Sequences. RNA samples were completely digested either by 0.2 M NaOH (37°, 18 hr) or by a mixture of RNases A, T1, and T2 (20). Nucleotides were separated by paper ionophoresis at pH 3.5. Ten percent polyacrylamide gels were prepared and run as described (21). Bromphenol blue was the tracking dye; *Dictyostelium* 4S and 5S RNAs and reovirus oligo(A) (33) were standards. After electrophoresis, gels were sliced into 1.7-mm sections and solubilized in scintillation fluid.

Poly(dT) Sequences in Dictyostelium DNA. ^{32}P-Labeled *Dictyostelium* nuclear DNA was depurinated by the diphenylamine procedure (23). After ether extraction, the poly(dT) sequences were purified by adsorption to and elution from poly(A)–Sepharose.

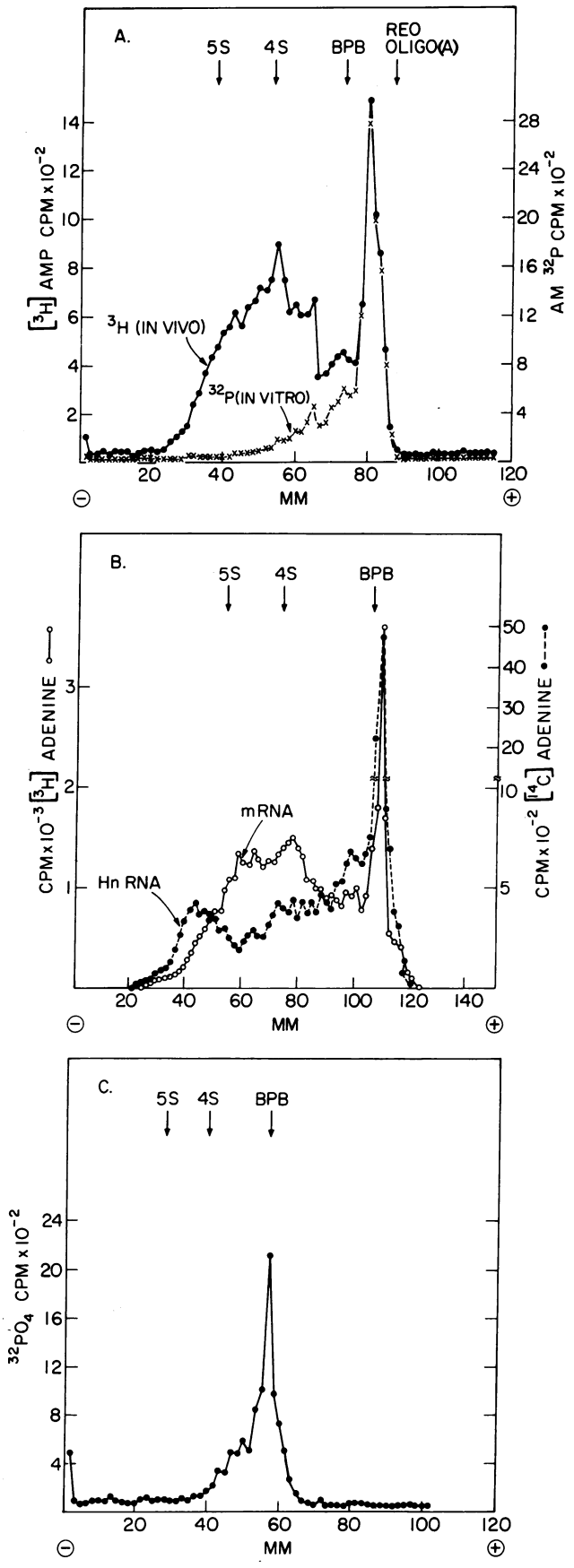

FIG. 1. Analysis of poly(A) and poly(dT) on polyacrylamide gels. (*A*) mRNA labeled in whole cells with [^{3}H]adenine (16) was mixed with mRNA precursor labeled in isolated nuclei (at 0.25 M KCl) with [α-^{32}P]ATP (5 Ci/mmol) (17) and digested with RNases A and T1 in 0.30 M NaCl–0.030 M Na citrate for 30 min at 37°. Poly(A) was purified and subjected to electrophoresis in a 10% polyacrylamide gel. *BPB*, position of bromphenol blue tracking dye. (●) ^{3}H-labeled poly(A) of mRNA; (×) poly(A) labeled with ^{32}P in isolated nuclei. (*B*) ^{14}C-labeled nuclear and ^{3}H-labeled cytoplasmic adenine-labeled heterogeneous RNAs were isolated by poly(U)–Sepharose chromatography. After digestion with RNase A and RNase T1, poly(A) was purified by oligo(dT)–cellulose chromatography and subjected to electrophoresis in 10% polyacrylamide gels. (*C*) Cells of *Dictyostelium discoideum* were labeled in MES-HL5 medium (16) with 50 mCi of $^{32}PO_4^{-3}$. Nuclear DNA was purified (22) and chemically depurinated (23). After depurination and ether extraction, the DNA was chromatographed on poly(A)–Sepharose. Poly(dT) residues, bound to and eluted from poly(A)–Sepharose, were precipitated with ethanol and then subjected to electrophoresis on a 10% gel. Values on *ordinate* have been multiplied by 10^{-2} or 10^{-3}, as indicated.

RESULTS

Poly(A) Sequences in mRNA and mRNA Precursor. We previously reported that *Dictyostelium* mRNA contains sequences of poly(A) with a mean size of 100 nucleotides (15). Such sequences migrate on 10% polyacrylamide gels between 4S and 5S RNA markers (Fig. 1*A*). Fig. 1 shows that mRNA also yields a second class of poly(U)-selected segments—presumably poly(A) sequences—migrating just in front of the bromphenol blue dye. By extrapolation from the mobilities of 4S and 5S RNA and that of reovirus oligo(A), about 16–18 bases long (Fig. 1*A*), we calculate that this species contains about 25 bases. Oligonucleotides from an unfractionated RNase digest of RNA migrate on these gels considerably faster than the presumptive poly(A)$_{25}$ (30). Digestion with alkali of poly(A)$_{100}$ derived from ^{32}P-labeled mRNA yielded 99.8% AMP, showing that indeed it is poly(A). Since no significant amount of non-adenylate nucleotides was obtained, this must mean that the poly(A)$_{100}$ is at the 3′ end of the mRNA. By contrast, digestion of the presumptive poly(A)$_{25}$ yielded 97.2% A, 1.4% U, 0.7% C, and 0.7% G. From this composition we calculate that the latter are poly(A) sequences on the average 34 bases long, terminated at the 3′ end by a U, C, or G residue; the error in this calculation is large since only a small amount of radioactivity above background was recovered in the nonadenylate nucleotides.

Dictyostelium mRNA has an average molecular weight of 400,000 (1300 bases) of which 44% (570 bases) are adenylate. After RNase T1 plus A digestion and poly(U)–Sepharose chromatography, 20% of the radioactivity in [^{3}H]adenosine-labeled mRNA is recovered in poly(A) sequences (see also refs. 15 and 16). Of the poly(A), 75% is of size 40–150 nucleotides (20 to 70-mm fractions) and 25% is in the smaller poly(A)$_{25}$ region (Fig. 1*A*). Hence it appears that each mRNA contains, on the average, one sequence each of poly(A)$_{25}$ and poly(A)$_{100}$. This calculation is only approximate, since we are using only average values for the molecular weight of the mRNA and of the poly(A).

In *Dictyostelium* nuclei there is a poly(A)-containing RNA of average molecular weight 500,000 which is the material precursor of mRNA (16), Nuclear poly(A)-containing RNA also contains two size classes of poly(A) (Fig. 1*B*): one migrates with the poly(A)$_{25}$ isolated from mRNA; the second is larger, including some molecules slightly larger than the poly(A)$_{100}$ found in mRNA. Based on calculations similar to those with mRNA, we conclude that, on the average, each molecule of mRNA precursor contains one sequence of poly-

$(A)_{25}$ while only about one molecule in three contains longer poly(A) sequences.

Isolated *Dictyostelium* nuclei synthesize, *in vitro*, poly(A)-containing RNA with size and other physical properties indistinguishable from the mRNA precursor isolated from pulse-labeled cells (17). These nuclei synthesize the $poly(A)_{25}$ species, but make no poly(A) sequences of larger size (Fig. 1*A*). Alkaline digestion of poly(A) synthesized by nuclei using [α-^{32}P]ATP yielded over 98% labeled AMP. Hence, the nearest neighbors of the vast majority of the AMP residues are AMP; this is further evidence that the material is poly(A).

Location of Both $Poly(A)_{100}$ and $Poly(A)_{25}$ at the 3′ End of mRNA:

(*1*) In isolated nuclei, under conditions permitting only the addition of 200–300 nucleotides to nascent RNA chains, all labeled RNA molecules that bind to immobilized poly(U) contain a $poly(A)_{25}$ tract; these molecules are identical in size to the mRNA precursor labeled in whole cells (17). RNA that does not adhere to poly(U) filters contains no detectable $poly(A)_{25}$ sequences. Were the $poly(A)_{25}$ located at a position other than the 3′ end, then it would be expected that a significant fraction of the poly(U)-binding RNA would consist of molecules of less than "complete" length.

(*2*) Cytoplasmic poly(A) containing RNA from ^{32}P-labeled cells was digested with either RNase T1 or RNase A, and the resultant poly(U)-selected sequences were analyzed on parallel polyacrylamide gels (Fig. 2). The poly(A)-rich sequences resulting from RNase A digestion resembled that obtained by the combined action of RNases A and T1 (compare to Fig. 1*A*): sequences both 100 nucleotides and 25 nucleotides long were obtained. Poly(A)-rich sequences obtained from RNase T1 digestion were considerably larger—about 150 nucleotides long—and relatively less $poly(A)_{25}$ was produced. The poly (A)-containing sequences containing 100–200 nucleotides from the T1 digest were eluted from the gel and redigested with RNase A (Fig. 2*B*). The bulk of the material migrates with the $poly(A)_{100}$ sequences produced by RNase A alone (compare with Fig. 2*A*) and, significantly, $poly(A)_{25}$ sequences are also liberated. Since we know that the $poly(A)_{100}$ sequences are at the 3′ end of mRNA, the above experiment demonstrates that in at least half of all mRNA molecules the $poly(A)_{25}$ sequences are no farther than 150–200 bases from the 3′ end of the mRNA. Redigestion with T1 RNase of the $poly(A)_{100}$ liberated initially by RNase A did not yield a significant amount of $poly(A)_{25}$ sequences (Fig. 2*B*). These data show that both types of poly(A) sequences are found at the 3′ end of mRNA. $Poly(A)_{100}$ appears on RNA molecules after $poly(A)_{25}$ (Fig. 1*A* and *B*; Firtel, Jacobson, and Lodish, in preparation). This is consistent with the notion that $poly(A)_{100}$ is at the 3′-OH end of mRNA and that the $poly(A)_{25}$ precedes it.

Poly(dT) Sequences in Dictyostelium DNA. One interpretation of the above results is that the $poly(A)_{25}$ species are encoded in the nuclear DNA and are transcribed by RNA polymerase, whereas the longer poly(A) sequences would be added post-transcriptionally. That *Dictystelium* DNA does contain poly(dT) sequences of approximate size to encode the $poly(A)_{25}$ was indicated by the following. RNA was transcribed from *Dictyostelium* DNA by *E. coli* RNA polymerase using [α-^{32}P]ATP and three other nonradioactive nucleoside triphosphates. Six percent of this RNA was $poly(A)_{25}$ (30). Further, ^{32}P-labeled *Dictyostelium* nuclear DNA was depurinated, and poly(dT) sequences were selected for by poly(A)–Sepharose

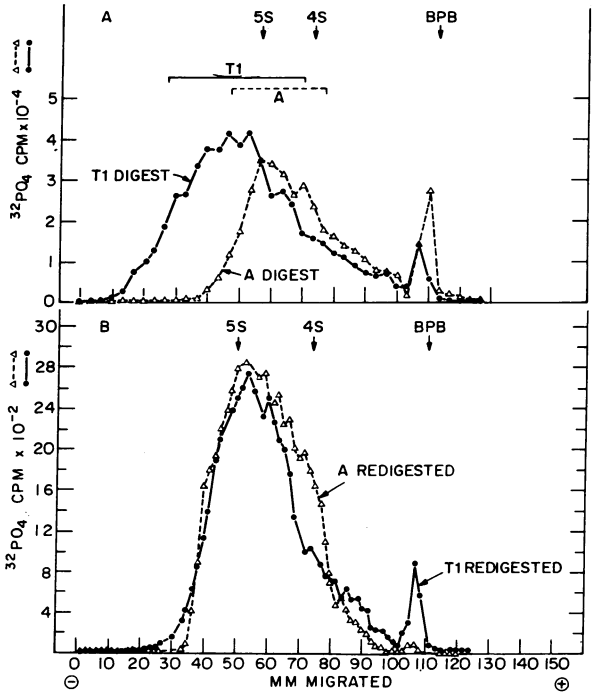

FIG. 2. Poly(A)-rich sequences produced from mRNA by digestion with either RNase A or RNase T1. (*A*) Cytoplasmic poly(A)-containing RNA isolated from cells labeled with $^{32}PO_4^{-3}$ for 1.3 hr was divided into two parts and digested for 1 hr with either RNase A (10 μg/ml) or RNase T1 (10 μg/ml). After chromatography on poly(U)–Sepharose, the poly(A)-rich oligonucleotides were analyzed on parallel 10% polyacrylamide gels. The gels were slices and counted by Cerenkov radiation. Poly(A)-rich sequences from: (●——●) RNase T1 digest; (Δ- - -Δ) RNase A digest. (*B*) The fractions containing large poly(A)-rich nucleotides liberated by RNase T1 or A digestion were pooled as indicated by the *brackets* in *A*. RNA was eluted from the gel. The poly(A)-containing sequences liberated by RNase T1 were then redigested with TNase A, and those liberated by RNase A were redigested with RNase T1. Resulting poly(A) sequences were purified by poly(U)–Sepharose chromatography and analyzed on parallel 10% polyacrylamide gels. Poly(A) sequences produced by redigestion with: (●——●) RNase A of the poly(A)-rich sequences liberated from mRNA by RNase T1; (Δ- - -Δ) RNase T1 of poly(A)-rich sequences liberated from mRNA by RNase A.

chromatography. In this fraction was 0.3% of the DNA radioactivity. These presumptive poly(dT) sequences migrate with virtually the same mobility as do the $poly(A)_{25}$ sequences in mRNA (Fig. 1*C*). This material was further characterized by two-dimensional "homochromatography" (20, 24). The material migrated in positions indicating that it was 20–25 nucleotides long and contained over 95% TMP residues. The base composition was confirmed by analysis of the products of digestion with alkaline phosphatase followed by venom phosphodiesterase. We conclude that this represents sequences of $poly(dT)_{25}$ in the genome.

Hybridization of Poly(A) and Poly(U) to Nuclear DNA. Denatured *Dictyostelium* DNA was hybridized to an excess of [^{3}H]poly(A) or [^{3}H]poly(U) (Table 1). Poly(A) hybridized to 0.33% of nuclear DNA while poly(U) hybridized to 0.57% of the DNA. The expected value for poly(U) would be twice (0.66) that of poly(A), since the conditions of hybrid formation strongly favor $dA \cdot rU_2$ triple helixes (25). The experimental value is lower than expected possibly because of the

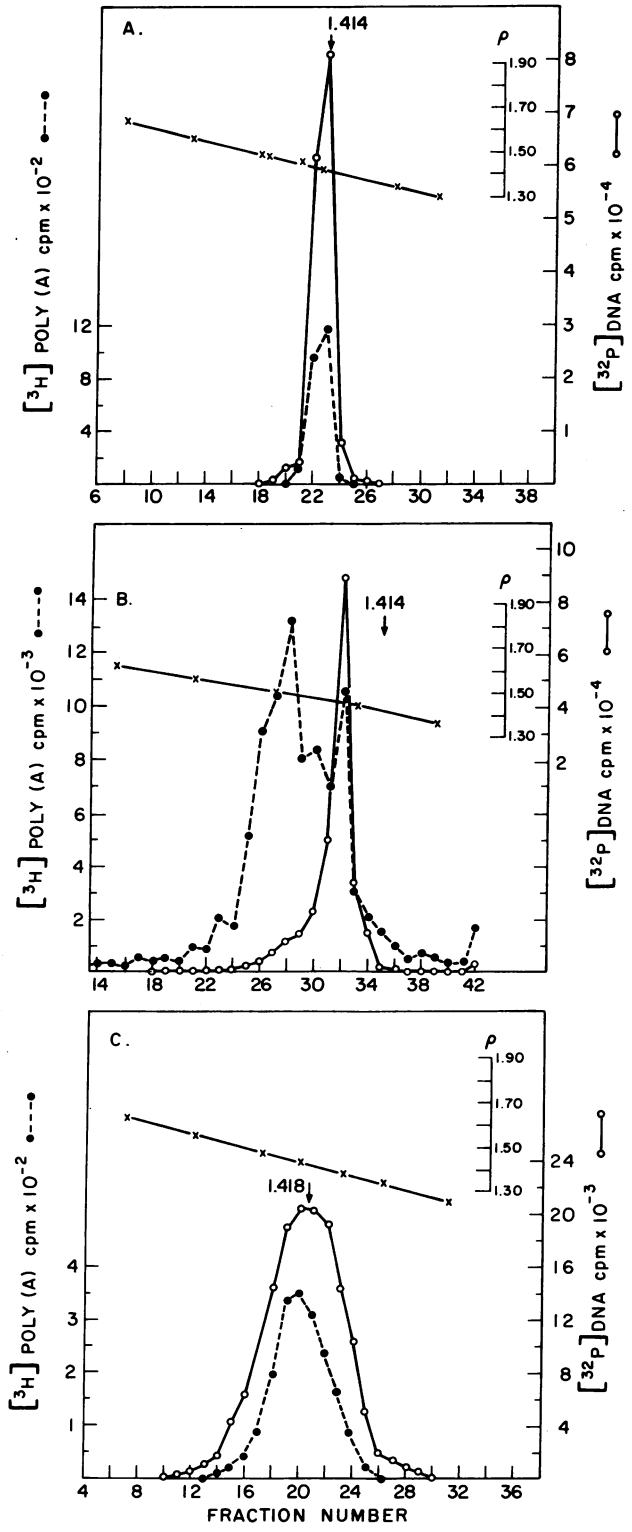

FIG. 3. Cs_2SO_4 bouyant density centrifugation of hybrids of [^{3}H]poly(A) and *Dictyostelium* [^{32}P]DNA. *Dictyostelium* [^{32}P]-DNA (8000 cpm/μg) was hybridized to an excess of [^{3}H]poly(A) (36,000 cpm/μg) in 0.5 ml (see *legend* to Table 1). The individual samples were treated as described below and banded in 10 ml of Cs_2SO_4 gradients, pH 7.0 (50 mM HEPES–NaOH) in a Spinco 65 rotor at 36,000 rpm for 60 hr at 16°. Fractions were dripped from the bottom. The nucleic acids were precipitated with trichloroacetic acid and collected on Millipore filters. The density was calculated from refractometer readings. (*A*) Unsheared DNA. Hybrids were treated with 1 unit/ml of RNase T2 before centrifugation (see *legend* to Table 1). (*B*) Unsheared DNA. No RNase treatment. (*C*) DNA sheared to 350 nucleotides before hybridization. Hybrids were treated with 1 unit/ml of RNase T2 before centrifugation (see *legend* to Table 1).

partial sensitivity of poly(U)·dA hybrids to RNase A. In a control experiment hybridizing poly(dA) to an excess of [^{3}H]poly(U), we recovered in the hybrid only 1.8 U residues per dA residue. As a further control, bacteriophage λ DNA does not hybridize to either poly(A) or poly(U). From the data in Table 1 and the nuclear genome size of *Dictyostelium* (30 to 35 × 10^9 daltons, ref. 22) we calculate that 3.6 × 10^5 TMP residues per genome are in dT·rA hybrids. This is equivalent to 15,000 sequences of poly(dT)$_{25}$ per genome.

The DNA·[^{3}H](rA) hybrid showed a very broad thermal melting profile with a Tm of 45° (R. Firtel, unpublished), which is 17° below the value of 62° obtained for long hybrids of poly(dT)·poly(rA). This is consistent with the notion that the sequences of hybrids of poly(dT)·poly(A) are very short (29), although this result, in itself, would be consistent with mismatching. Since the amount of poly(dT) in *Dictyostelium* DNA was the same (0.3%) whether determined by depurination (Fig. 1*C*) or hybridization (Table 1), we believe there is no extensive mismatching in the poly(A)·poly(dT) hybrids.

Interspersion of Poly(dT) Sequences in Nuclear DNA. The poly(dT) sequences are interspersed throughout the DNA and are not clustered in a particular region of the genome. Unsheared DNA (average single-strand molecular weight 5 × 10^6) and sheared DNA (molecular weight 100,000) were hybridized with [^{3}H]poly(A), treated with RNase T2 [to remove unhybridized poly(A)], and then centrifuged to equilibrium in a Cs_2SO_4 gradient (Fig. 3). The [^{3}H]poly(A)·DNA hybrid is found at the same density as the majority of DNA. [In DNA sheared to 350 bases, the hybrid has a slightly higher density than DNA, since 30 residues of hybridized [^{3}H]-poly(A) will increase slightly the density of DNA to which it is bound.] If the poly(dT) sequences were clustered in either long or short stretches, then one would expect to obtain with both sheared and unsheared DNA a [^{3}H]poly(A)·DNA satellite band at a density near that of RNA·DNA hybrid of density 1.45 g/cm^3. The following experiment also suggests that the poly(dT) sequences are interspersed throughout the DNA. Denatured DNA was hybridized to an excess of [^{3}H]-poly(A) and the mixture was banded in Cs_2SO_4 without prior nuclease treatment (Fig. 3*B*). The density of essentially all of the DNA was now considerably greater than that of DNA alone. Presumably this is due to the [^{3}H]poly(A) attached to each DNA molecule. [The ^{3}H band at ρ = 1.52 is free poly-(A).] The sharpness of the peak of labeled DNA implies that the frequency of the poly(dT) stretches—and hence the amount of poly(A) bound per DNA length—is uniform. Similar results were obtained if poly(dA) was hybridized to *Dictyostelium* DNA and then banded in CsCl or Cs_2SO_4. Control experiments using poly(A) hybridized to lambda DNA showed no [^{3}H]poly(A) under the DNA peak after T2 digestion and no shift in the density of the lambda DNA if T2 nuclease was not used.

DISCUSSION

The mRNA of *D. discoideum* apparently contains two discrete species of poly(A) segments. One, about 25 nucleotides long, is transcribed from a poly(dT)·poly(dA) region in DNA and a second, which averages 100 nucleotides long, is presumably added post-transcriptionally. The amount of poly-

TABLE 1. *Hybridization of poly(A) and poly(U) to Dictyostelium DNA*

Deoxypolymer	[^{3}H] Ribopolymer	Ribopolymer hybridized (μg)	Fraction of Ribopolymer to deoxypolymer in hybrid
Dd DNA (10 μg)	Poly(A)	0.033	0.0033
Poly(dT) (0.3 μg)	Poly(A)	0.29	0.97
Dd DNA (10 μg)	Poly(U)	0.057	0.0057
Poly(dA) (0.3 μg)	Poly(U)	0.55	1.8
λ DNA (10 μg)	Poly(A)	<0.003	—
λ DNA (10 μg)	Poly(U)	<0.003	—

Specific activity of poly(U) and poly(A) = 36,000 cpm/μg. Reactions contained DNA or deoxyhomopolymer and 1 μg of the [^{3}H]ribohomopolymer. Hybridization was performed in 0.36 M Na^+ (pH 7) for poly(A) and 1.2 M Na^+ for poly(U) at 50° for 90 min; mixtures were allowed to cool slowly to room temperature. Nuclease digestions were at 25° and 0.30 M Na^+ for poly(A) and at 0.5 M Na^+ for poly(U) for 30 min. RNase A (2 μg) at pH 7.3 was used to digest unhybridized poly(U) and 1 μg of T2 RNase, pH 6.0, was used to digest unhybridized poly(A) in a final volume of 1 ml. *Dd DNA, D. discoideum* DNA.

$(A)_{25}$ and $poly(A)_{100}$ recovered from mRNA is such that each mRNA molecule could contain one sequence of each. As this calculation assumes an average size of mRNA of 400,000 daltons and average sizes of the poly(A) sequences, it is subject to some error. Since mRNA yields both large and small poly(A) tracts after nuclease treatment, large poly(A) could not be formed by continuing the polymerization of an already existing poly(A) tract. Large and small poly(A) must be minimally separated by a UMP, a CMP, or a GMP residue. The experiment in Fig. 2 shows that in at least half the mRNA molecules, the $poly(A)_{25}$ sequences are separated by no more than about 25 bases from the $poly(A)_{100}$ at the 3′ end of mRNA. From the data of Fig. 1*A* and *B*, we infer that the $poly(A)_{100}$ sequence is not immediately added to the mRNA precursor after synthesis, but at some time before it exits from the nucleus. Since isolated nuclei synthesize only $poly(A)_{25}$ but not $poly(A)_{100}$ (Fig. 1*A*), it is likely that they are defective in the enzymatic activity responsible for adding $poly(A)_{100}$ (17).

The location of a $poly(A)_{25}$ sequence at or near the 3′-OH end of virtually all mRNA transcripts suggests it may be a recognition site for the enzyme that adds $poly(A)_{100}$ post-transcriptionally. Alternatively a cluster of dA·dT base pairs in DNA may be a genetic signal for the termination of transcription by RNA polymerase. It should be noted: (*1*) that three prokaryotic RNAs whose synthesis terminates *in vitro* without factors other than RNA polymerase and DNA have the sequence $-UUUUUUA_{OH}$ (26, 32) or $-UUUUUUAA_{OH}$ (27) at their 3′ ends and (*2*) that putative gene-sized pieces of DNA of the protozoan *Stylonychia* are rich in dA·dT base pairs at one end (31).

Almost all *Dictyostelium* mRNA contains some poly(A). The recovery of 14,000–15,000 $poly(dT)_{25}$ sequences per nuclear genome agrees with previous estimates for the number of *Dictyostelium* mRNAs (28). The hybridization experiments show that the poly(dT) sequences are interspersed throughout the genome and are not clustered in one region. Thus, it is likely that most DNA sequences coding for mRNA (informational DNA) terminate with poly(dT). It is also likely that the informational DNA is randomly located on both DNA strands, since hybridization between poly(A) and large, unsheared DNA molecules shifts the density of all DNA molecules (Fig. 3).

This research was supported by Grant No. GB-29308 from the National Science Foundation. A.J. is a fellow of the Jane Coffin Childs Memorial Fund for Medical Research. R.A.F. is a fellow of the Helen Hay Whitney Foundation. H.F.L. is a recipient of a Research Career Development Award no. 1 KO4 GM 50175 from the U.S. National Institutes of Health. We thank Ms. Lolley Gee for expert technical assistance. We also thank Dr. Aaron Shatkin (Roche Institute) for providing the reovirus oligo(A) marker.

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