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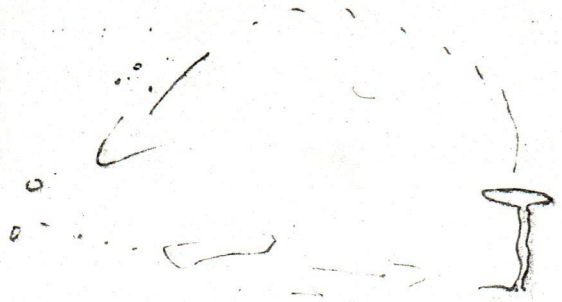

ELECTROPHORETIC SEPARATION OF THE MAJOR SPECIES OF SLIME MOLD MESSENGER RNA

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INTRODUCTION

As a response to starvation, amoebae of the cellular slime mold, Dictyostelium discoideum, will form spores. The formation, and subsequent dissemination, of such spores is engineered by the construction of multicellular fruiting bodies. In this process, groups of approximately 10^5 amoebae aggregate and ultimately differentiate into either spore cells or stalk cells (2,22,26). Morphologically, and biochemically, vegetative amoebae differ significantly from either spore or stalk cells. During the transition from amoeba to differentiated cell gene expression is carefully regulated, i.e., it has been shown that many Dictyostelium genes are expressed at specific "developmental" stages (1,4,6,8,13-15,27-29 A). The evidence to support this notion is the following:

(1) Gamma irradiation of amoebae can impair the ability of amoebae to propagate without impairing their ability to aggregate and develop (4).

(2) A number of enzymes show dramatic increases in specific activity at discrete developmental stages. For at least two of these enzymes, the increase in specific activity can be accounted for by de novo synthesis of enzyme protein; further, these changes in enzyme activity appear to require prior RNA synthesis (8,13-15,27,28).

(3) RNA-DNA hybridization experiments demonstrate that approximately half of the non-reiterated portion of the genome is transcribed throughout the entire course of the life cycle. Of the RNA transcribed, half appears to be present in cells of all developmental stages, whereas half can be subdivided into stage-specific subclasses (6).

(4) Mutants have been isolated which are defective in the ability to grow vegetatively, but which have unimpaired developmental capacities. The reciprocal

types of mutants have also been isolated. The frequencies with which the two classes arise suggest that perhaps 20 times as many genes are required for vegetative growth as are required for gross aspects of development (25,27). (5) Experiments in which cells have been pulse-labeled with radioactive amino acids at discrete stages of the life cycle have shown that a number of polypeptides (identified as "bands" on polyacrylamide gels) are preferentially labeled at specific developmental stages (24,29). The specific labeling patterns are qualitatively similar even in very short pulses of amino acids (1). Moreover, these changes in the labeling patterns of polypeptides can be reproduced by cell-free extracts capable only of completing nascent polypeptide chains (1). These two results strongly suggest that preferential protein synthesis is the mechanism responsible for the observed changes. Preliminary experiments, in which purified RNA has been added to RNA-dependent cell-free protein synthesizing extracts, have shown that RNA of a specific stage can direct the in vitro synthesis of polypeptides characteristic of that stage (1).

In short, a significant number of independent experiments demonstrate that different genes are expressed at different stages of the slime mold life cycle. Moreover, it has been shown that these changes in gene expression could be accounted for, at least in part, by alterations in the transcription of the genome. This by no means demonstrates that changes in RNA synthesis are the controlling element in slime mold development, but it does suggest that this process may well be important. Clearly, a thorough analysis of the structure and synthesis of Dictyostelium messenger RNA should provide insight into the nature of the mechanisms operative in transcriptional control.

Such studies have been initiated (6,8,10,11,12,16-19,23,24). Characterization of total poly(A)-containing RNA has yielded information on the

organization and transcription of an "average" gene and pointed to the need to extend such studies to individual RNA species. In this paper, we discuss the synthesis and processing of an "average" messenger RNA and then describe our recent results on the fractionation of slime mold mRNA.

STRUCTURE AND TRANSCRIPTION OF AN "AVERAGE" GENE

Figure 1 summarizes our current knowledge on gene structure and mRNA processing in D. discoideum. The data supporting this model have been discussed at length elsewhere and shall only be briefly described here (6,8,10,11,12,16-19 23,24).

Most of the messenger RNA in Dictyostelium, and other eukaryotes, contains poly(A) (3,10,17,18,30). Thus, unless otherwise specified, the term messenger RNA refers to RNA molecules purified by virtue of their affinity for immobilized poly(U) or oligo(dT). Dictyostelium mRNA is found on polyribosomes, has a DNA-like base composition, and an average size of 400,000 daltons (10,12,18,23). It will program the synthesis of authentic Dictyostelium polypeptides in cell-free extracts (24). Further, such mRNA renatures to excess DNA with kinetics indicating that 90% of its nucleotide sequences have been transcribed from unique (single copy) DNA and 10% from repetitive DNA (10-12,23).

The precursor to messenger RNA is a nuclear poly(A)-containing RNA that is only 20-25% longer than mRNA (10,11,23). There is no evidence for the existence of longer poly(A)-containing RNAs in the nucleus, e.g., molecules analogous to the HnRNA of metazoan cells (3,30). DNA complementary to messenger RNA will hybridize to 70% of nuclear poly(A)-containing RNA (23). Thus, this species is an informational precursor to mRNA. Further, 70% of pulse-labeled nuclear poly(A)-containing RNA can be "chased" into mRNA (12,23). Thus, this RNA is also the material precursor to mRNA. In addition to being

slightly longer than mRNA, the mRNA precursor differs from mRNA in its kinetics of reassociation to excess DNA: 25-30% of its nucleotide sequences renature to repetitive DNA and the remaining renature to unique DNA (11,12,23). The repetitive and unique transcripts appear to be covalently linked and the repetitive sequences appear to occur predominantly in the 300-350 nucleotides adjacent to the 5' terminus of the RNA (12,23). It is this region of the molecule that is apparently preferentially lost during the maturation of the precursor.

Messenger RNA and its precursor also differ with respect to the nature of their poly(A) tracts (11,17). Messenger RNA contains equimolar amounts of two classes of poly(A): $poly(A)_{100}$ and $poly(A)_{25}$ (11,17,18). The two are closely linked at the 3' end of mRNA, with the $poly(A)_{100}$ occupying the 3' OH terminus. The two classes of poly(A) are, however, separable by at least one non-A nucleotide ("X" in Figure 1) (17). The mRNA precursor contains preferentially $poly(A)_{25}$. mRNA precursor labeled in isolated nuclei contains only $poly(A)_{25}$ (11,17,18). These data, and others, suggested that $poly(A)_{25}$ may be transcribed whereas $poly(A)_{100}$ would be added post-transcriptionally. This was confirmed by an analysis of the poly(dT) tracts of Dictyostelium DNA (17). Such DNA contains 14-15,000 sequences of $poly(dT)_{25}$, but no larger poly(dT) (17). The $poly(dT)_{25}$ tracts could code for $poly(A)_{25}$; they are interspersed throughout the genome and occur at the same frequency as the known total number of expressed genes (6,17). The function of $(dA:dT)_{25}$ tracts in DNA is unknown, but it is speculated that they may represent transcription termination signals.

The interspersion of (dT) sequences throughout the genome, the association of these sequences with informational DNA, and the precursor-product relationship

of nuclear and cytoplasmic poly(A)-containing RNAs permit the construction of the model described in Figure 1 (23,24). R, R', and R" and SC, SC', and SC" represent different repetitive and single copy sequences, approximately 300-350 and 1000-1100 nucleotides long, respectively. The unit R-SC-A_{25}X represents the initial transcript by RNA polymerase, with X the non-A nucleotides. Prior to transport to the cytoplasm, the longer poly(A) sequence is added to this molecule post-transcriptionally and the majority of the repetitive sequences are removed from the 5' end. The timing of the latter events is not definitive. Approximately 70-75% of the sequences in nuclear poly(A)-containing RNA are conserved and transported to the cytoplasm, while the remainder is presumably destroyed in the nucleus.

Since only 50-60% of the genome has been shown to be transcribed over the bulk of the life cycle (6), we have left spacer units between the primary transcription units although there is no direct evidence for such spacers. This arrangement of nucleotide sequences is consistent with an analysis of the interspersion of repetitive and single copy DNA sequences in the Dictyostelium genome (9,11).

Again, we must stress that the data on mRNA structure and synthesis is drawn from the average behavior of all mRNAs and must be extended to specific mRNA species. A method for the detection and isolation of the major species of Dictyostelium mRNA is discussed in the following section.

FRACTIONATION OF SLIME MOLD MESSENGER RNA

Polyacrylamide gel electrophoresis of the total protein component of any cell will reveal, upon staining, a number of discrete "bands." These correspond to the major cellular polypeptides, i.e., those polypeptides present in the greatest abundance. We reasoned that the messenger RNA for many abundant poly-

peptides may also be abundant and, thus, we predicted that on the appropriate gel system it should be feasible to recognize "bands" of messenger RNA. Figure 2 demonstrates that this assumption is valid. In this experiment ^{32}-P-labeled, oligo(dT) cellulose-purified, polysomal RNA was electrophoresed on a 4.4% gel containing 99% formamide. Formamide was included to completely denature all RNA molecules and thus eliminate any effects of secondary structure on electrophoretic migration (5). After electrophoresis, the wet gel was subjected to autoradiography. Figure 2 shows that a large number of high molecular weight RNA "bands" can be resolved by this procedure. It should be noted that the method used for oligo(dT) cellulose chromatography is one which deliberately permits some contamination of the messenger RNA preparation with 28S and 18S rRNA (16). The presence of these RNAs on a gel provides convenient markers. Figure 3 is a densitometer tracing of the same gel. It can be seen that the "bands" represent a significant fraction of the total RNA and, further, that the "bands" are present over a heterogeneous background. This background undoubtedly represents the thousands of mRNAs present at very low concentrations.

The conditions used for electrophoresis in the experiment of Figure 2 did not provide adequate resolution near the top of the gel. Figure 4 demonstrates that such resolution can be obtained by using longer times of electrophoresis and lower percentage gels (3.75%).

The evidence that the radioactivity in "bands"corresponds to radioactivity in messenger RNA can be summarized as follows: 1) The "bands" are associated with polyribosomal RNA; 2) At low concentrations (2-5 ug/ml), Actinomycin D will preferentially inhibit labeling of Dictyostelium ribosomal RNA (8); gels of RNA labeled in the presence of ActD show diminished labeling of only the 28S and 18S "bands (and their two precursors), but no others (20); 3) Extensive

purification of the RNA on oligo(dT) cellulose or poly(U) sepharose eliminates only ribosomal RNA "bands from a sample (20) ; and 4) RNA can be eluted from a band, rebound to oligo(dT) cellulose or immobilized poly(U) and rerun to its characteristic position on a gel. Elution, rebinding, and rerunning of the RNA in 14 different bands yields the expected diagonal when the samples are re-electrophoresed in 14 different slots of a slab gel (Figure 5). If the RNAs did not contain poly(A), or were partially degraded fragments of rRNA or mRNA, this result could not be obtained.

Figure 6 shows the efficiency with which RNA eluted from a band will rebind to poly(U) filters. For most RNAs, rebinding occurs with at least 80% efficiency. Rebinding of RNA in the vicinity of 18S and 28S rRNA shows depressed binding.

Further evidence that the RNA in a band was mRNA was obtained from experiments demonstrating that eluted RNA could stimulate cell-free protein synthesis (20). For such experiments to be interpretable, it was necessary to show that virtually all of the RNA in a given region of the gel was the same as the RNA recognizable as a band. To this end, it was shown that labeling times of 15 minutes, one hour, or four hours gave RNA with qualitatively identical band patterns on a gel (20). Since Dictyostelium mRNA has a half-life of 3½-4 hours (7), we were thus satisfied that the unlabeled RNA in a gel band was not different from the labeled species. Thus, it was significant to find that eluted RNA could stimulate cell-free protein synthesis in wheat germ extracts. This not only confirmed the notion that the RNA in the bands was mRNA, but it also facilitated the identification of the polypeptides coded for by different mRNA bands. For example, Figures 7 and 8 are autoradiograms of 10% polyacrylamide gels of the polypeptides synthesized in vitro when wheat germ extracts are

programmed with RNA eluted from gels. For convenience, we characterize an RNA by its electrophoretic migration relative to 18S rRNA (R_{18S}). Several points are worth noting: 1) The extracts do not efficiently translate mRNAs larger than 1800-2000 nucleotides; 2) The spread of translation activity for a given polypeptide is greater than the breadth of any radioactive band; 3) The polypeptides coded for by eluted RNA correspond in molecular weight to major polypeptides labeled in whole cells; 4) Smaller RNAs direct the synthesis of smaller polypeptides, but there is a substantial range of sizes of polypeptides coded for by any one class of mRNA; 5) Many eluted RNAs are substantially pure with respect to the polypeptides that they code for. It is particularly worth noting (see Figure 8) that in one step it is possible to obtain an RNA almost 50% pure with respect to actin messenger activity. It should be emphasized that this may be a low estimate because it assumes that none of the "bands" on the protein gel is a fragment of actin, i.e., "early quitters." In fact, it is quite likely that many of these are actin fragments. We should also note that substantially purer preparations of actin mRNA can be obtained if the eluted RNA is rerun on another gel or if the RNA is prefractionated on formamide-sucrose gradients before running on a gel (20).

In summary, fractionation of mRNA on formamide-acrylamide gels will resolve a number of bands. These appear to be the mRNAs for major cellular polypeptides including the mRNA for actin. Further purification of these mRNAs will permit us to extend our studies of mRNA metabolism to individual message species. The gels provide an excellent diagnostic method for detecting major changes in the mRNA population or for recognizing specific species of mRNA precursor. Using these methods, we have obtained similar results with RNA of other eukaryotic organisms (21). However, we have not successfully resolved

RNA bands using other gel systems (20). In particular, we have found that non-denaturing gels are especially unsuitable.

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FIGURE LEGENDS

Figure 1. A Model of Dictyostelium Nuclear DNA.

The primary genetic unit in Dictyostelium contains a repetitive DNA sequence (R) of average 300-350 nucleotides at the 5' end, a sequence of non-reiterated or single-copy (SC) DNA of 1100-1200 nucleotides, and a sequence of 25 adenylic acid residues (A_{25}) at the 3' end. This unit is transcribed by RNA polymerase and then poly(A) of 100-150 residues is added after transcription. Before transport of the heterogeneous RNA to the cytoplasm, the majority of the 5' repetitive sequences are removed, leaving a short repetitive sequence. The evidence for the presence of a portion of the repetitive sequence transcript (r) on the majority of mRNA molecules is given in References 11 and 12. Since the $poly(A)_{25}$ and $poly(A)_{100}$ are separable on polyacrylamide gels after digestion with RNase T_1 and RNase A, at least one other nucleotide (X) must be present between the 3' end of the short $poly(A)_{25}$ and the 5' end of the larger $poly(A)_{100}$ (17).

Figure 2. Electrophoretic Separation of the Major Species of Slime Mold mRNA.

Log phase amoebae of strain AX3 were concentrated to 10^7 cells/ml in MES-HL5 medium supplemented with 3 mC/ml of $^{32}PO_4$ (16). After 60 minutes of labeling, RNA was isolated from polyribosomes and purified by chromatography on oligo(dT) cellulose (16). Columns were only washed with binding buffer to permit recovery of some ribosomal RNA in the eluate. RNA eluted from the column was ethanol

precipitated, resuspended in sterile water, and lyophilized in siliconized glass tubes. The dry powder was resuspended in sample buffer (5,16) and electrophoresed on 4.4% polyacrylamide slab gels containing 99% formamide (5,16). Electrophoresis was in an E.C. Apparatus slab gel box (E.C. Apparatus, St. Petersburg, Florida) at 100 V for 16 hours. After electrophoresis, the gel was removed from the box, placed on a glass plate, covered with Saran Wrap, and then exposed to Kodak Royal Blue x-ray film for approximately 10 hours. The two samples shown represent two aliquots of the same sample.

CBF is the position of the xylene cyanole ff tracking dye. This dye routinely migrates in this gel system as if it were a molecule approximately 250 nucleotides long.

Actin mRNA denotes the band which migrates in the position of actin messenger activity (see Figures 7 and 8).

Figure 3. Densitometer Tracing of an RNA Gel.

An autoradiogram of the gel shown in Figure 2 was scanned with an E.C. Apparatus densitometer. The inset shows the gel.

Figure 4. RNA Fractionation on Lower Percentage Gels.

RNA was labeled in vegetative amoebae as described in the Legend to Figure 2, and subsequently isolated from whole cells. Further purification, electrophoresis, and autoradiography were the same as in the experiment of Figure 2 with the following exceptions: the gel concentration was 3.75% and electrophoresis was for 20 hours at 100 V.

Figure 5. Electrophoresis of RNA Eluted from a Gel and Repurified on Oligo(dT) Cellulose.

An autoradiogram was used as a template to excise portions of a gel corresponding to "bands." A gel comparable to those of Figures 2 and 4 was used.

RNA was eluted from gel slices and repurified by oligo(dT) cellulose chromatography (16). Subsequently, 14 different samples were re-electrophoresed in 14 different slots of a 3.75% slab gel. The two outer slots contained total mRNA. Electrophoresis and autoradiography were as in Figure 2.

Figure 6. Quantitation of Elution and Rebinding.

The experiment of Figure 5 was repeated. Radioactivity eluted was monitored by 1) counting aliquots in aquasol (total cpm), 2) assaying acid-precipitable radioactivity (TCA filter cpm), and 3) assaying binding to poly(U) immobilized on glass fiber filters(cpm poly(U) filter) (16).

Figure 7. Translation of Eluted RNA in Wheat Germ Cell-Free Extracts.

The gel of Figure 4 contained a total of 100-200 ug of mRNA. Portions of this gel were excised, and the RNA eluted and repurified on oligo(dT) cellulose as described in the Legend to Figure 5. Aliquots of 0.2-0.5 ug of purified RNA were translated in wheat germ extracts containing ^{35}S-methionine (24). The total *in vitro* product was layered on 10% polyacrylamide slab gels containing 0.1% SDS and subjected to electrophoresis and autoradiography as described (24).

The numbers 0.1-2.1 are the mobilities of a given RNA relative to that of 18S rRNA (R_{18S}). The slots beneath these numbers correspond to the *in vitro* products whose synthesis was programmed by these RNAs.

The following controls are included: 1) Two wheat germ reactions lacking added RNA (NO RNA); 2) The products of a wheat germ reaction programmed with *total* cytoplasmic RNA from vegetative amoebae (Veg. Cyto. RNA); 3) The total polypeptides labeled in vegetative amoebae after a two-hour incubation with ^{35}S-methionine (Veg.); and 4) The products of a wheat germ reaction programmed with *total* RNA from cells at two hours of development (2 HR. RNA).

The position of the prominent actin band is noted (29).

Figure 8. Further Translation of Eluted RNA.

This experiment is identical to that of Figure 7 with two exceptions: 1) mRNA was isolated from cells at two hours of development, and 2) More fractions were taken in the region of actin mRNA activity.

Controls: 2 HR. CELLS - total polypeptides from cells labeled with ^{35}S-methionine from 0-2 hours of development; 2-2.5 HR. CELLS - total polypeptides from cells labeled with ^{35}S-methionine from 2.0-2.5 hours of development.

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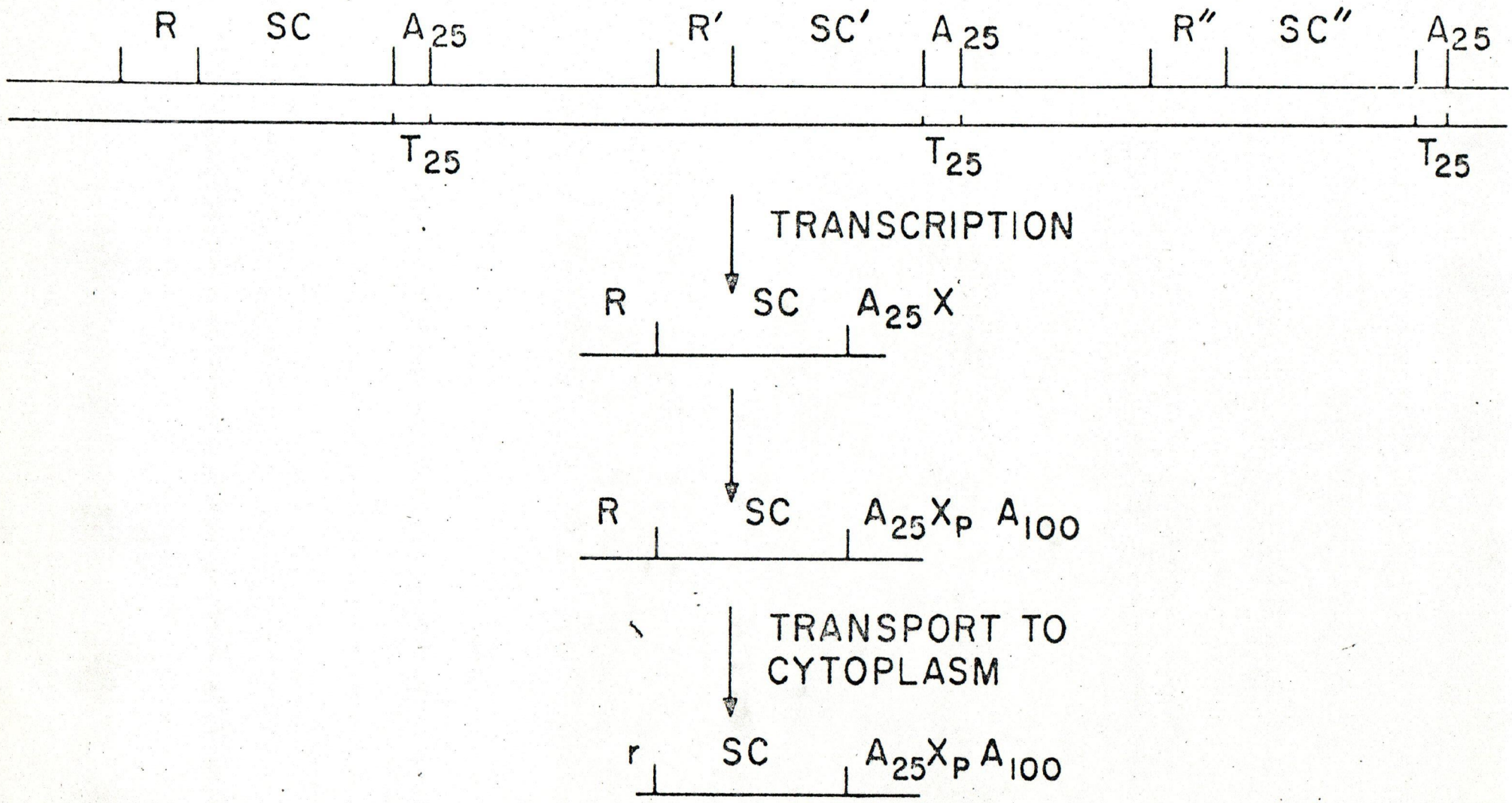
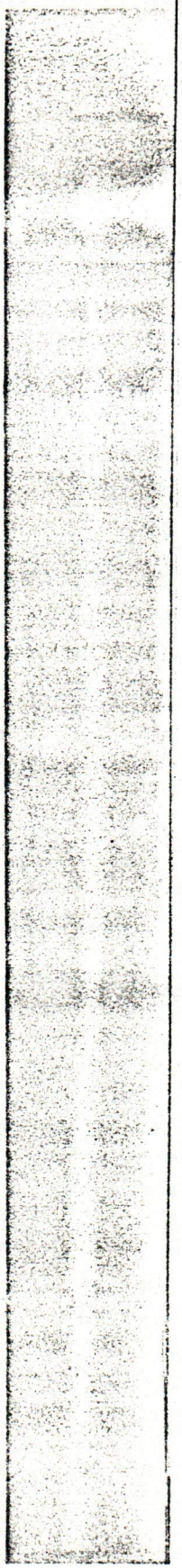
STRUCTURE AND TRANSCRIPTION OF DICTYOSTELIUM DNA





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— Origin

— 28S rRNA

— 18S rRNA

— Actin mRNA

— CBF

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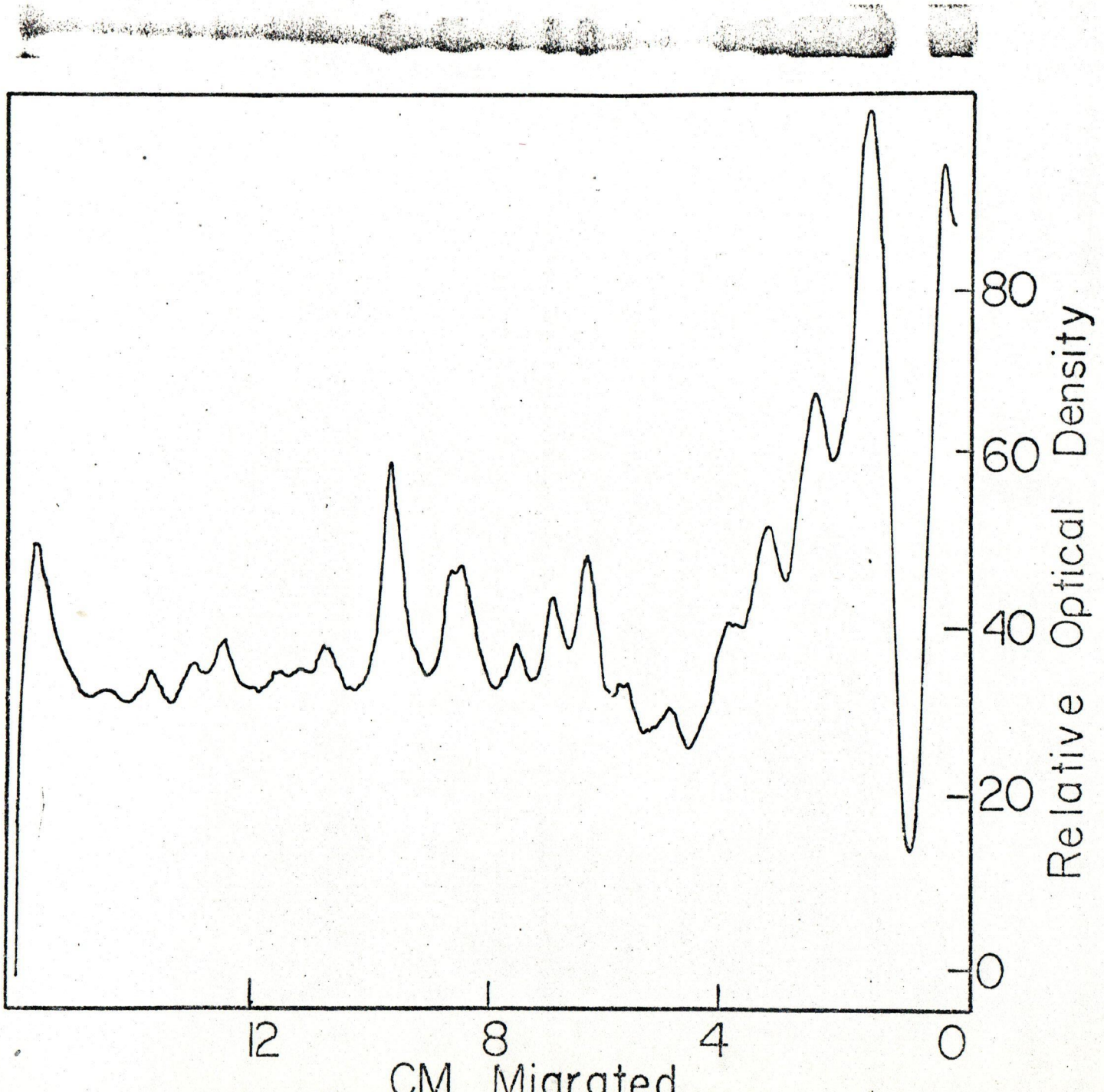
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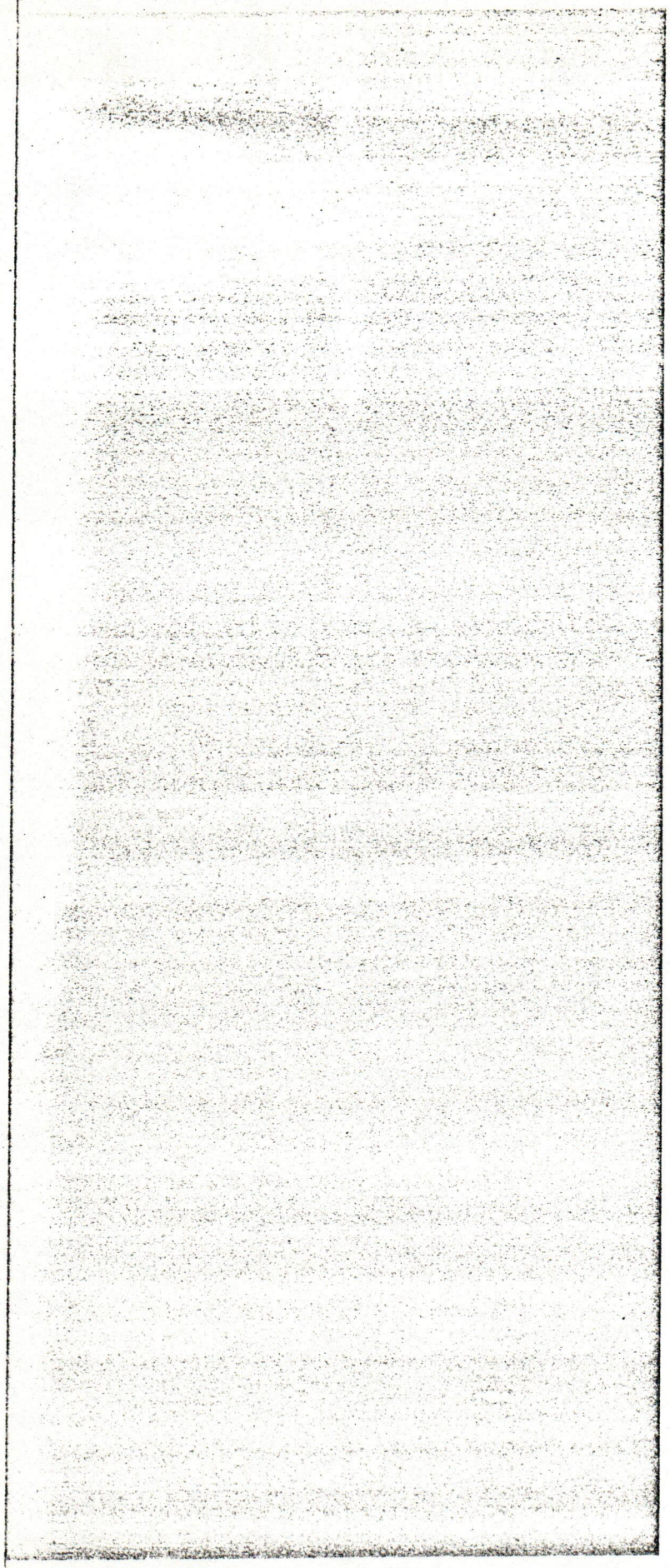
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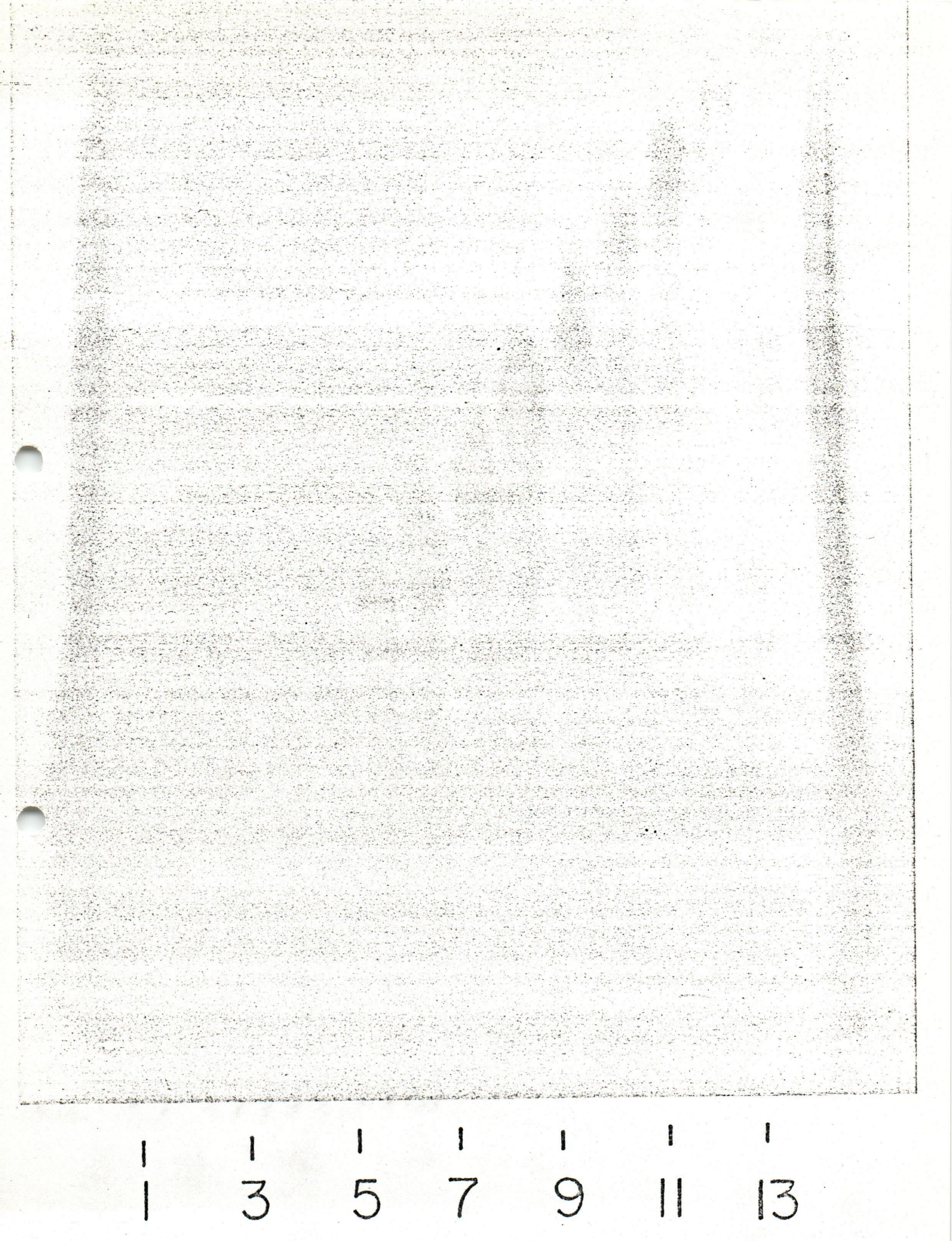
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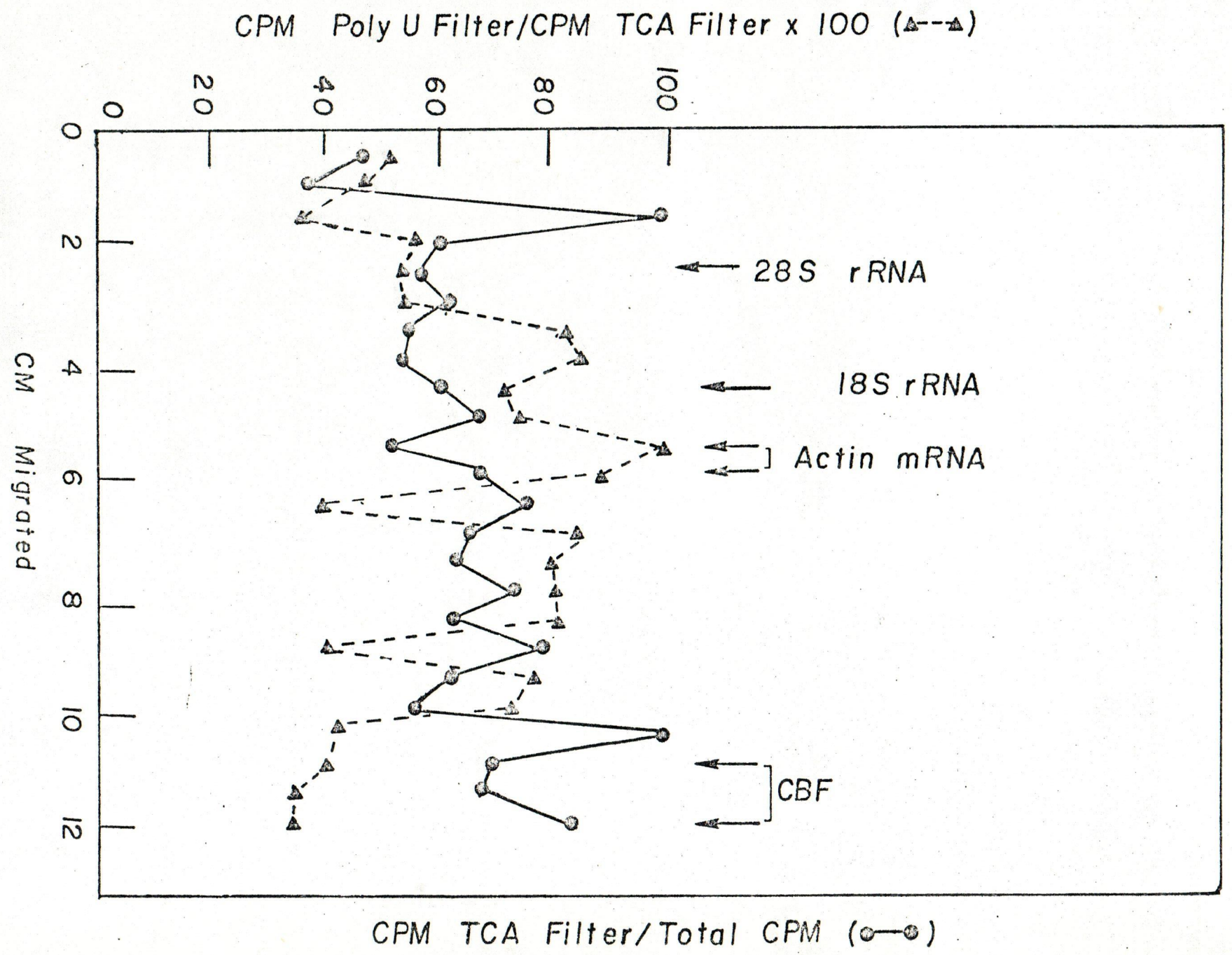
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