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Transcription and polyadenylation processes during early development of quail embryo

By B. OLSZAŃSKA[1], B. KLUDKIEWICZ[2] AND Z. LASSOTA[2]

From the Institute of Genetics and Animal Breeding and Institute of Biochemistry and Biophysics of the Polish Academy of Sciences

SUMMARY

Transcription of tRNA and mRNA occurs in quail as early as during cleavage while rRNA transcription becomes measurable during blastulation. The polyadenylated fraction content in newly synthesized RNA amounting to 6 % during cleavage and blastulation decreases to 3 % during gastrulation. Up to 3/4 adenylic residues incorporated into RNA during cleavage are accumulated in the polyadenylated molecules mainly in the form of RNAse-resistant tracts.

ABBREVIATIONS

SDS – sodium dodecylsulphate
TRIS – tris (hydroxymethyl) aminomethan
TCA – trichloroacetic acid
PPO – 2,5-Diphenyloxazole
POPOP – 1,4-bis [2-(5-phenyl)oxazolyl] benzene
EDTA – ethylene [diamine] tetraacetate
PIPES – 1,4-piperazinediethane sulphonic acid

INTRODUCTION

In embryo massive transcription of genes coding for major classes of RNA does not start immediately after fertilization nor does it begin simultaneously for all classes. Despite the delayed onset of transcription of the genome, early development proceeds utilizing the stock of maternal RNA accumulated in the oocyte. Such a general picture differing in many details has emerged from studies on early embryogenesis of Echinoderms, Amphibia and Mammals (see Davidson 1976, for review). For birds, however, no basic data concerning RNA synthesis and polyadenylation during early embryogenesis are available, except the report on rRNA synthesis occuring in chick embryo before gastrulation (Wylie, 1972).

The aim of this work was to gain insight into transcription and polyadenylation during avian embryogenesis beginning from cleavage.

[1] *Author's address:* Institute of Genetics and Animal Breeding of the Polish Academy of Sciences. Jastrzebiec 05-551, Poland.

[2] *Authors' address:* Institute of Biochemistry and Biophysics of the Polish Academy of Sciences. Warszawa 02-532, Rakowiecka 36, Poland.

MATERIALS AND METHODS

Material

Japanese quails (*Coturnix coturnix japonica*) were reared at the experimental farm of the Institute of Genetics and Animal Breeding in Jastrzebiec.

The embryo of quail undergoes cleavage and blastulation in the oviduct. The state of the uterine egg envelope is a convenient index of the advancement of embryogenesis (Olszańska & Stepińska, 1983). In eggs with a soft egg membrane the blastoderms are at cleavage stage while in those with a hard calcareous egg shell they are at blastulation. Gastrulation occurs in the deposited eggs when they are incubated at an appropriate temperature. When classified according to Hamburger-Hamilton's tables (Hamburger & Hamilton, 1951), quail embryos at the beginning of incubation (*in ovo* at 38 °C) are at stage 1, i.e. gastrulation, and after 1 day of incubation most of them are at stage 6, i.e. postgastrulation.

Uterine eggs removed from the quail oviduct without sacrificing the birds, as described for hen (Eyal-Giladi & Kochav, 1976), were segregated into batches with soft and hard egg envelopes. The deposited eggs collected and stored at 12–15 °C for maximally 4 days until use were incubated at 38 °C, which allowed the embryos to reach stages 1–4 or 6–10.

In vitro *culture*

Embryos at cleavage (about 30 cells), blastulation (about 5000 cells), gastrulation and postgastrulation were used. They were isolated together with a piece of adhering vitelline membrane on a filter-paper ring and were cultured *in vitro* on egg albumen as described before (Olszańska & Lassota, 1980, Olszańska, Szolajska & Lassota, 1983). The blastoderms from uterine eggs were cultured at 40–41 °C, and those from the deposited eggs at 38 °C. The culture medium was supplemented with 20–100 μCi/ml of either 5,6-[^{3}H]uridine (spec. act. 44 or 46 Ci/mmol), or 8-[^{3}H]guanosine (spec. act. 7·7 Ci/mmol) or else 2-[^{3}H]-adenosine (spec. act. 22 or 23 Ci/mmol), all from the Radiochemical Centre, Amersham, U.K.

The efficiency of DNA and RNA labelling

The efficiency of labelling was measured in embryos cleaned of the adhering yolk and albumen. Embryonic tissues suspended in 0·01 M-Tris-1 % SDS were solubilized by twice repeated digestion (for 1 h at 37 °C) with pronase (selfdigested for 3 h at 37 °C, 40 μg/ml). Then, pronase was inactivated for 5 min at 100 °C and nucleic acids were precipitated with 2·5 vol. of 96 % ethanol in the presence of 0·4 M-NaCl. DNA was removed from the precipitate dissolved in 0·01 M-Tris (pH-5), by twice repeated digestion (for 20 min at 37 °C) with DNase free of RNAases (Worthington, U.S.A., 10 μg/ml). Before and after RNAase treatment, radioactivity was measured in the TCA-precipitable material on Whatman

GF/C filters in PPO-POPOP-toluene scintillant in a Beckman scintillation counter. From these data the percentage of the DNAase-resistant fraction corresponding to labelled RNA was calculated.

RNA isolation

RNA was isolated from the embryos by hot phenol-SDS extraction at pH 5 (Olszańska, Grabczewska & Lassota, 1974), with additional treatment of the interphase with a phenol–chloroform mixture (1:1). DNA was removed by twice repeated digestion of the sample (for 10 min at 37 °C) with DNAase free of RNAase (Worthington, U.S.A. 10 μg/ml) (Knowland, 1970). Polyadenylated RNA was separated by affinity chromatography on oligo/dT/cellulose (Aviv & Leder, 1972), with three repeated recycling of the effluent before washing the column with the binding buffer.

Separation of RNA on Sephadex column

RNA was separated into the light (4–5 S) and heavy ($\geqslant$8·8 S) fractions on Sephadex G-100 in an aqueous system (10 mM-NaCl, 1 mM-EDTA, 2·5 mM-PIPES, 7·5 mM-Tris pH 7·6) according to Duncan & Humphreys (1981). The column was calibrated with 8·8 S (Miles, U.S.A.) and 4–5 S RNA (Sigma, U.S.A.). The RNA fractions were precipitated with ethanol from the respective pooled fractions, as indicated above.

RNAase digestion

The RNAase-resistant core was isolated from poly/A/$^+$RNA labelled with [^{3}H]adenosine. To the sample containing 10–20 μg RNA in 1 ml of low-salt buffer (0·1 M-NaCl), RNAases A and T_1 (Sigma, U.S.A.) were added in the amounts of 2·5 μg/ml and 5 i.u./ml, respectively, and digestion was carried out for 30 min at 37 °C, according to Jelinek & Darnell (1972). The digest was deproteinized with a phenol–chloroform mixture, and the RNAase-resistant core was precipitated with ethanol, as mentioned above. *E. coli* sRNA was used as carrier.

Gel electrophoresis

Electrophoresis of poly/A/$^+$RNA was performed in 3·5 % polyacrylamide gel under denaturing conditions in formamide (Staynov, Pinder & Gratzer, 1972), formamide (Fluka, Switzerland) being purified by shaking with Amberlite MB-1 for 2 h. Electrophoresis of poly/A/$^-$RNA was run in 2·4 %. polyacrylamide gel under normal conditions (Bishop, Claybrook & Spiegelman, 1967), the RNAase-resistant core was electrophoresed in 7·2 % polyacrylamide gel under normal as well as under denaturing conditions using 4 S and 5 S RNA as markers. The gels stained with methylene blue were cut into 1 mm slices which were solubilized in 30 % H_2O_2 at 60–70 °C. The radioactivity of samples was

measured in a Beckman scintillation counter with PPO-POPOP-dioxane scintillant.

RESULTS

Synthesis of rRNA, tRNA and poly/A/$^+$RNA

The labelling of RNA with radioactive uridine has been reported to be ineffective in early vertebrate embryo. In case of mammalian embryo this fact has been attributed to the low permeability of its cells to the precursor (Clegg & Pikó, 1977), while for the avian embryo utilization of the uridine pool for DNA synthesis has been reported (Emanuelson, 1966). Our attempt to label RNA in the cleaving embryo with tritiated uridine failed mainly because of the substantial incorporation of the isotope into the DNAase-digestible material (Table 1). Tritiated guanosine was more effective than uridine for labelling RNA at cleavage. The synthesis of RNA at later developmental stages was readily measurable after incubation with tritiated uridine or tritiated adenosine (Table 2).

Table 1. *Efficiency of RNA labelling with [^{3}H]uridine in early quail embryo*

Embryos derived from eggs:	^{3}H-label incorporated into DNAase-resistant material (% of total incorporation)
uterine, in soft egg envelope	48
uterine, in soft calcified shell	51
uterine, in hard shell	89
freshly laid	95

Six to nine embryos cultured for 7 h *in vitro* on 2 ml of egg albumen containing 100 μCi/ml of 5,6-[^{3}H]uridine (spec.act. 46 Ci/mmol).

Table 2. *^{3}H-radioactivity incorporated into total RNA during 7 h of* in vitro *culture**

Developmental phase	[^{3}H]uridine labelling† c.p.m./blastoderm	[^{3}H]adenosine-labelling‡ c.p.m./blastoderm
Cleavage	65–110 920§	1200–2700
Blastulation	4500–9500	11 000–23 500
Gastrulation	34 000	151 000–364 000
Postgastrulation	103 000	560 000–950 000

* The number of embryos per batch was 30–60 at cleavage and blastulation and 10–20 at gastrulation and postgastrulation cultured on 5 or 4 ml of egg albumen.

† [^{3}H]uridine was added in the amount of 100 μCi at cleavage and blastulation and of 50 μCi/ml at gastrulation and postgastrulation stages.

‡ [^{3}H]adenosine was added in the amount of 100 μCi/ml at all developmental stages.

§ [^{3}H]guanosine (instead of uridine) was added in the amount of 100 μCi/ml.

In the electrophoretic profile of poly/A/$^-$RNA synthesized at cleavage, the peak of tRNA was clearly visible; however, no peaks of rRNA were discernible (Fig. 1A). The transcription of rRNA genes only at blastulation yielded measurable quantities of radioactive 18 S and 28 S rRNA (Fig. 1B).

The synthesis of polyadenylated RNA seemed to be at least as effective at cleavage as during blastulation. After 7 h of labelling with radioactive uridine (or guanosine) poly/A/$^+$RNA accounted at both stages for about 6 % of newly synthesized RNA. At gastrulation and later the polyadenylated fraction amounted to 6–7 % of newly synthesized RNA after the first 2 h of incubation; however, after 7 and 19 h, poly/A/$^+$RNA accounted for 3 % and 2 % of total radioactive RNA, respectively (Table 3).

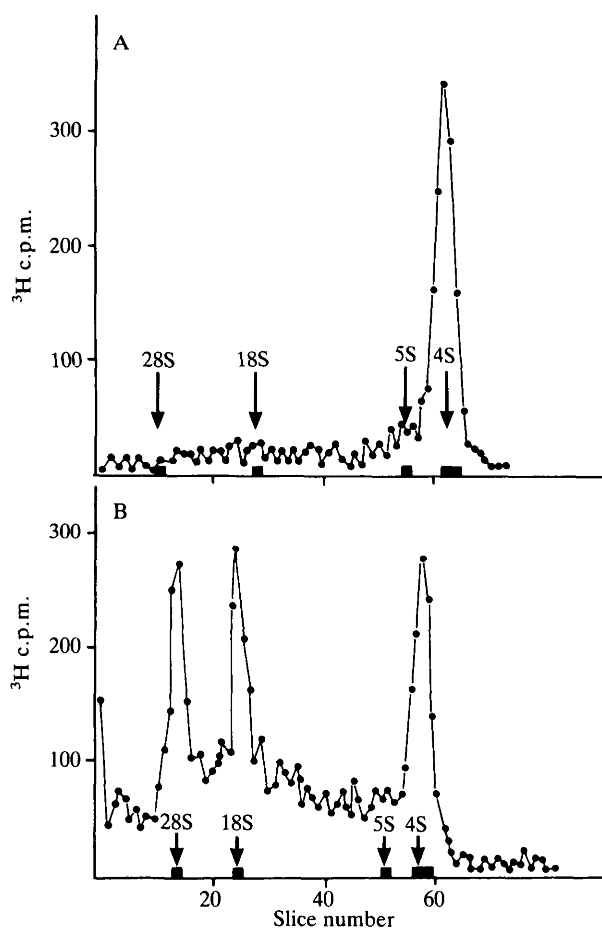

Fig. 1. Electrophoretic pattern of poly/A/$^-$RNA from early quail embryos. Embryos at cleavage (A) or at blastulation (B) were grown for 7 h *in vitro* in the presence of 100 μCi/ml of 2-[^{3}H]adenosine (spec. act. 22 Ci/mmol) and RNA was isolated and separated as described in Methods. Electrophoresis was run in 2·4 % polyacrylamide gel according to Bishop *et al.* (1972).

Table 3. *Poly/A/$^+$RNA content in RNA synthesized during early development of quail embryo (± S.D.)**

Developmental phase	Time of incubation with radioactive precursor (h)	Percentage of incorporated precursor found in poly/A/$^+$RNA fraction after labelling with	
		[^{3}H]uridine†	[^{3}H]adenosine‡
Gastrulation	1	6·9 ± 0·4 (2)	–
Postgastrulation	1	7·3 (1)	–
Gastrulation	2	6·3 ± 0·4 (5)	–
Postgastrulation	2	7·0 ± 0·0 (2)	–
Cleavage§	7	6·1 (1)	31·6 ± 5·8(5) 76·1 ± 0·1(2)
Blastulation	7	5·9 ± 0·7 (6)	10·6 ± 1·6(6)
Gastrulation	7	3·5 ± 0·5 (2)	6·7 ± 0·2(3)
Postgastrulation	7	3·2 (1)	7·2 (1)
Gastrulation	19	2·2 ± 0·15(2)	4·8 (1)
Postgastrulation	19	2·3 ± 0·2 (3)	5·4 (1)

* In brackets – number of experiments.

† The medium contained 5,6-[^{3}H]uridine (spec.act. 44 or 46 Ci/mmol) in the amount of: 20 μCi/ml during 1 h, 2 h and 19 h incubation at gastrulation and postgastrulation: 50 μCi/ml during 7 h incubation at gastrulation and postgastrulation: 100 μCi/ml during 7 h incubation at blastulation.

‡ The medium contained 2-[^{3}H]adenosine (spec.act. 22 or 23 Ci/mmol) in the amount of: 20 μCi/ml during 19 h incubation at gastrulation and postgastrulation; 100 μCi/ml during 7 h incubation at all tested developmental periods.

§ At cleavage, instead of uridine, 8-[^{3}H]guanosine (spec.act. 7,7 Ci/mmol) was present in the amount of 100 μCi/ml of medium during 7 h incubation.

Pre- and post-gastrular poly/A/$^+$RNA were heterogeneous and their electrophoretic patterns under denaturing conditions were similar (Fig. 2) both showing negligible radioactivity in the 4–5 S region.

Adenosine-rich poly/A/$^+$RNA

Marked differences depending on the stage of embryogenesis were shown in the polyadenylated RNA fractions when [^{3}H]adenosine was used to label the newly synthesized transcripts (Table 3). The relative poly/A/$^+$RNA content in total newly synthesized RNA, resulting from [^{3}H]adenosine incorporation, was considerably higher than that calculated from incorporation of [^{3}H]uridine. Doubling of the values obtained for blastulation and for later development may partly result from the fact that in the experiments with [^{3}H]uridine poly/A/ tracts remain unlabelled. However, at cleavage, the poly/A/$^+$RNA fraction accounted for as many as 30 % and in some cases even for 75 % of total [^{3}H]-adenosine-labelled RNA (Table 3).

The electrophoretic patterns of poly/A/$^+$RNA labelled with [^{3}H]adenosine

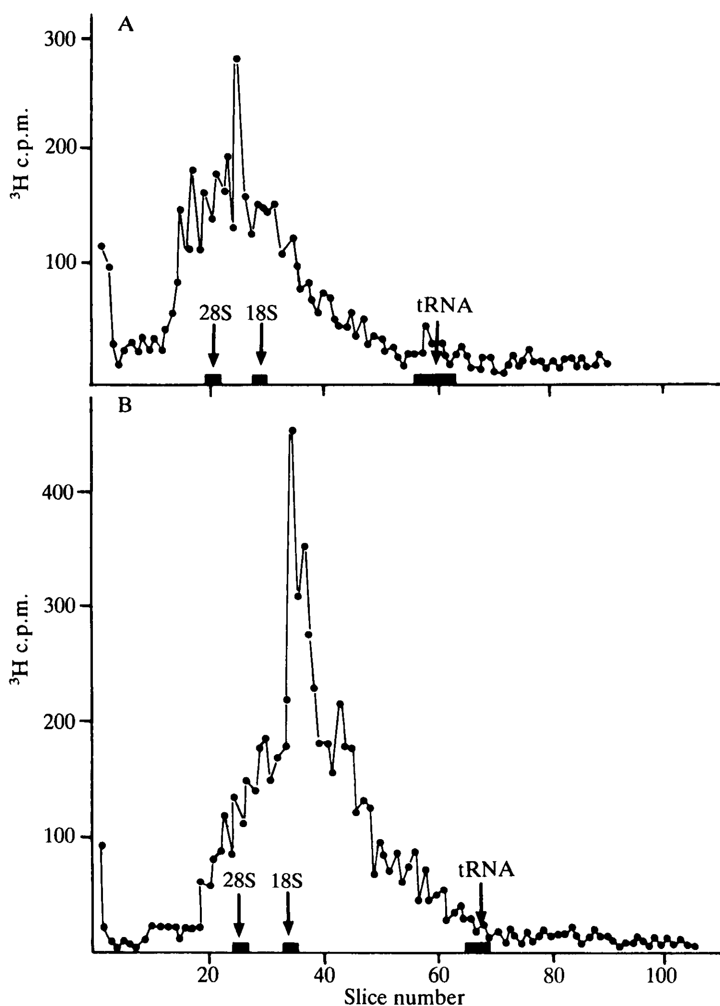

Fig. 2. Electrophoretic pattern of poly/A/$^+$RNA from early quail embryos labelled with [^{3}H]uridine. Embryos at blastulation (A) or postgastrulation (stages 6–10) (B) were grown for 7 h *in vitro* in the presence of 100 μCi/ml of 5,6-[^{3}H]uridine (spec. act. 46 Ci/mmol). RNA was isolated and separated as described in Methods. Electrophoresis was run in 3·5 % polyacrylamide gels in formamide according to Staynov (1972).

(Fig. 3) corroborated the heterogeneity observed for [^{3}H]uridine (Fig. 2), but no striking differences characteristic of the stage of embryogenesis were revealed.

The unusually high level of adenosine incorporated into poly/A/$^+$RNA during cleavage suggested a certain peculiarity of this process, as compared with later periods of quail embryogenesis. Thus, the localization, content and length of the homopolymeric /A/n tracts in this RNA fraction were investigated.

When polyadenylated RNA synthesized at cleavage in the presence of [^{3}H]-adenosine was separated into the heavy and light fraction on Sephadex G-100

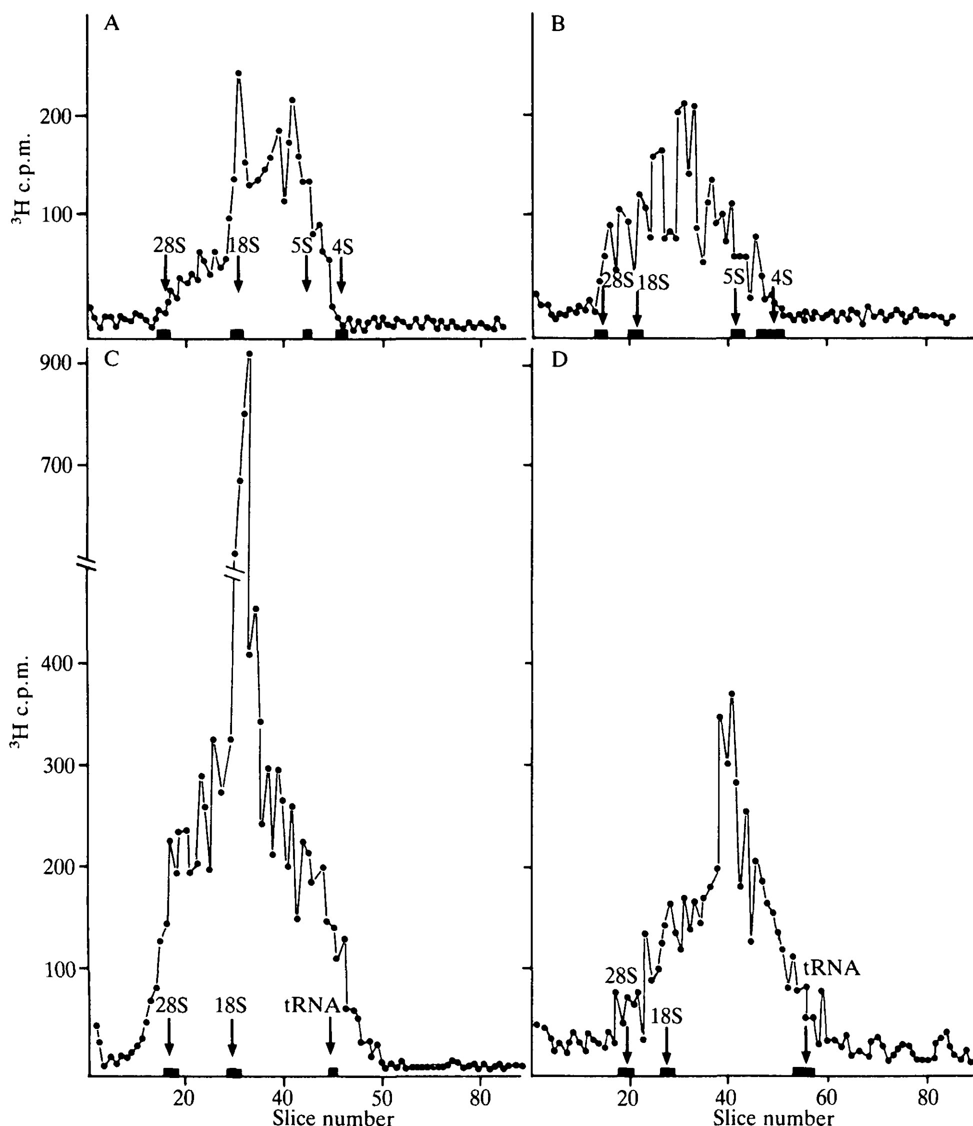

Fig. 3. Electrophoretic pattern of poly/A/$^+$RNA from early quail embryos labelled with [^{3}H]adenosine. The embryos were grown and RNA was separated as indicated in Fig. 1. Electrophoresis was carried out as in Fig. 2. (A) Embryos at cleavage, (B) embryos at blastulation, (C) embryos at gastrulation (stages 3–4), (D) embryos at postgastrulation (stages 6–10).

columns (Fig. 4, peaks I and II) from 155 000 c.p.m. put on the column 97 500 c.p.m. were recovered in the heavy and 44 400 c.p.m. in the light fraction. Out of the recovered 142 000 c.p.m., 29 % (41 060 c.p.m.), were in the oligo /dT/-bound material, this value corresponding well with the data shown in Table 3. The bulk of polyadenylated RNA, (39 700 c.p.m.) was present in the heavy

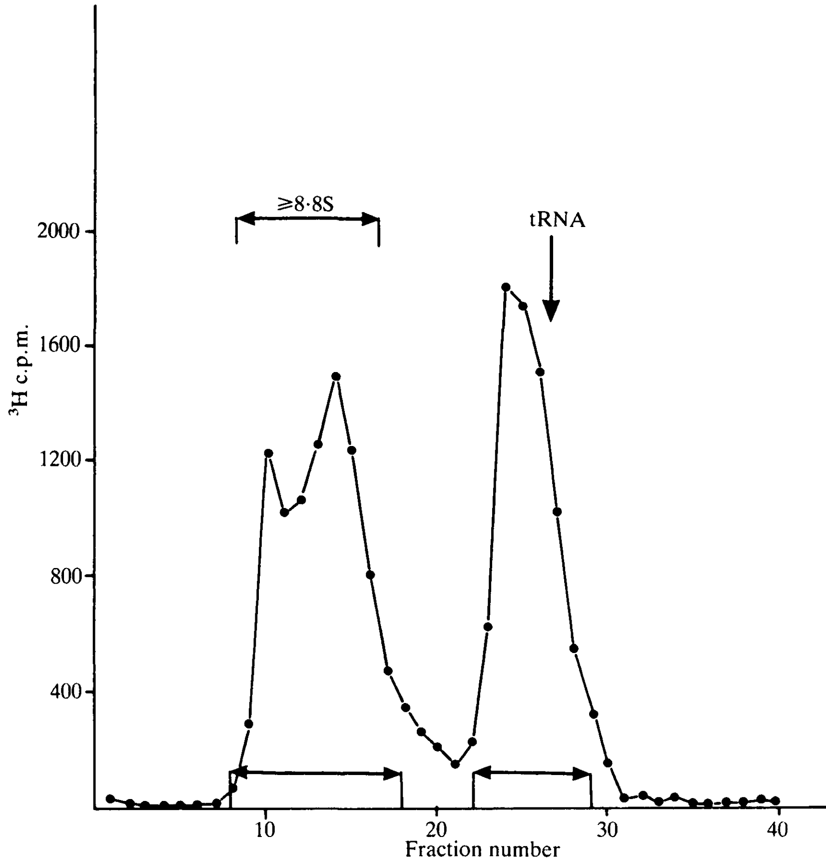

Fig. 4. Separation of RNA on a Sephadex column. Total RNA from 57 cleaving quail blastoderms labelled with [^{3}H]adenosine (100 μCi/ml of medium), *in vitro* for 7 h, has been applied on a Sephadex G-100 column in the amount of 155 000 c.p.m. Peak I – RNA ≥ 8·8 S, peak II – 4 to 5 S RNA.

RNA fraction and only 1360 c.p.m. were found in the light one. Thus, the adenosine-rich polyadenylated RNA chains arising at cleavage were longer than 8·8 S and did not differ in this respect from the average mRNA chains.

Poly/A/ and oligo/A/ tracts in RNA at cleavage

The percentage of [^{3}H]adenosine label located in the RNAase-resistant core of polyadenylated RNA amounted at cleavage to 80 % and decreased with the advancement of embryogenesis to 25 % after gastrulation (Table 4). Heat denaturation of the sample at 65 °C for 2 min before digestion with RNAases reduced the values obtained at cleavage to about 60 % and that at blastulation to 34 % indicating that the RNAase-resistant core contained along with $(A)_n$ tracts also denaturable double-stranded structures.

Table 4. *Incorporation of [^{3}H]adenosine into RNAase-resistant tracts of polyadenylated RNA during early development of quail embryo*

Developmental phase	Percentage of ^{3}H-label of poly/A/$^{+}$RNA found in RNAase-resistant tracts (± S.D.)
Cleavage	80·2 ± 1·25
Blastulation	42·1 ± 8·0
Gastrulation	
stage 1	34·9 ± 5·7
stage 3–4	28·8
Postgastrulation	
stages 6–10	25·7 ± 1·2
Control poly/A/$^{-}$RNA	5·2 ± 0·6

Embryos were grown *in vitro* for 7 h in the presence of 100 μCi/ml of 2-[^{3}H]adenosine (spec.act. 22 or 23 Ci/mmol).

The length of the RNAase-resistant fragments increased with embryonic development from less than 75 and maximally 120 nucleotides at cleavage (Fig. 5A, B) to 75–200 nucleotides at blastulation and later development (Fig. 5C–E).

It should be mentioned that at cleavage two different kinds of electrophoretic profiles of the RNAase-resistant fragments were observed for individual batches of blastoderms, and thus two electrophoretic patterns were presented in Fig. 5A, B similarly as two values of poly/A/$^{+}$RNA content are shown for this stage in Table 3.

DISCUSSION

The present results indicate that in quail embryo tRNA and poly/A/$^{+}$RNA are transcribed from the genome as early as cleavage in blastoderms consisting of up to 50 cells, while massive transcription of 18 S and 28 S rRNA genes becomes manifest only during blastulation in blastoderms with about 5000 cells and coincides with the formation of area pellucida. The time of appearance of newly synthesized rRNA, found for quail embryo, corresponds with the time of appearance of nucleoli recorded in chick embryo at stage VI of uterine development (Raveh, Friedlander & Eyal-Giladi, 1976). Measurable rRNA synthesis in chick embryo has also been observed at this period (Wylie, 1972).

The delayed start of rRNA synthesis suggests that a stock of rRNA and/or of ribosomes of maternal origin may be present in the avian oocyte at fertilization to supply the translation process necessary during cleavage. This assumption coincides with our observation that 1/3 of the tested quail embryos during (but not after) gastrulation resists the actinomycin block of rRNA synthesis (Olszańska & Kludkiewicz, 1983).

The avian embryo in which rRNA synthesis occurs at blastulation takes an

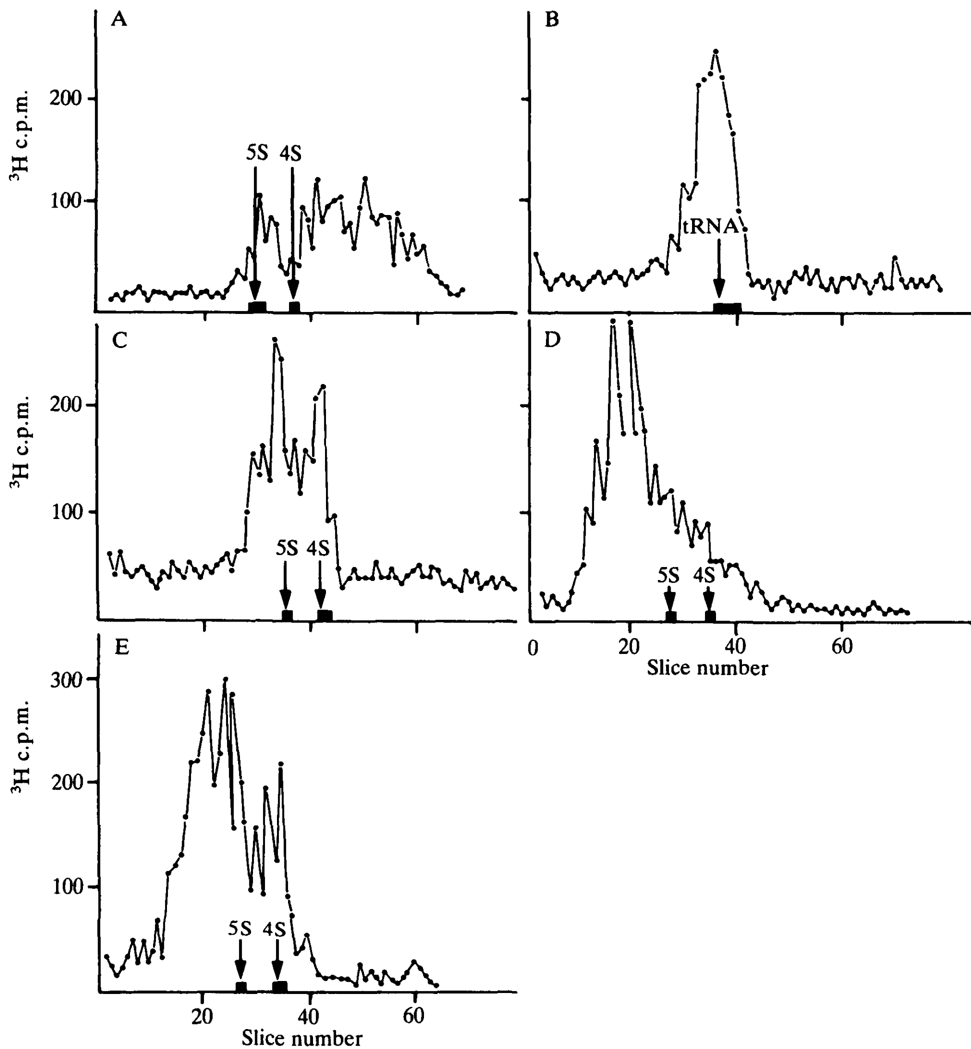

Fig. 5. Electrophoretic pattern of RNAase-resistant tracts of poly/A/$^{+}$RNA from early quail embryos labelled with [^{3}H]adenosine. Embryos were grown as indicated in Fig. 1. RNAase-resistant tracts were obtained as described in Methods. Electrophoresis was run in 7·2 % polyacrylamide gels according to Bishop *et al.* (1972). (A) and (B) embryos at cleavage, (C) embryos at blastulation, (D) embryos at gastrulation (stages 3–4), (E) embryos at postgastrulation (stages 6–10).

intermediate position between Mammals where this process starts at cleavage (Pikó, 1970; Clegg & Pikó, 1982; Pikó & Clegg, 1982), and Echinoderms and Amphibia where it begins with the onset of gastrulation (Davidson, 1976). This may be related to the fact that embryogenesis occurs in Mammals within the maternal organism, in Echinoderms and Amphibia it occurs externally, and in Aves it initially occurs within the maternal organism and thereafter beyond it.

Poly/A/$^+$RNA in quail embryo synthesized at cleavage comprises mainly molecules between 5–18 S. The analogous RNA fraction from mouse blastocysts found in 4–5 S region (Warner & Hearn, 1977), may be the result of RNA degradation, since poly/A/$^+$RNA from a 1-cell mouse zygote is located in the 18–28 S region (Clegg & Pikó, 1982). The content of poly/A/$^+$RNA at cleavage and at blastulation amounting to 6 % of newly synthesized RNA is high as compared with the values for gastrulation and later development (Table 3); however, in mouse egg polyadenylated RNA has been found to account for 8 % of RNA stock originating during oogenesis (Bachvarova & DeLeon, 1980).

A distinctive feature of polyadenylated RNA arising during cleavage consists of the strikingly high content of adenylic residues. The phase of cleavage seems to be metabolically differentiated and to include at least two populations of blastoderms in which 1/3 and 3/4 of the incorporated adenosine, respectively, are located in poly/A/$^+$RNA (Table 3). This is also reflected in the two different electrophoretic patterns of the RNAase-resistant core: one being heterogeneous, with prevalence of fragments shorter than 4 S (Fig. 5A), and another with a single peak formed by fragments slightly longer than 4 S (Fig. 5B). In sea urchin at cleavage the average length of the poly/A/tracts has also been found to vary from batch to batch from 20–40 to 70–120 nucleotides (Duncan & Humphreys, 1981).

Nevertheless, in the oligo/dT/-bound fraction of RNA from several batches of quail cleaving blastoderms, at least 60 % of radioactive adenylic residues form homopolymeric tracts (data from Table 4 corrected for denaturation). This high value, as compared with the length of RNA chains (Figs 3A and 4) and of $(A)_n$ segments (Fig. 5A, B), can hardly be explained in terms of simple *de novo* synthesis of poly/A/$^+$RNA.

Free segments consisting exclusively, or almost, of adenylic residues might, if present, result from specific cleavage of poly/A/$^+$RNA (Bergman & Brawerman, 1980), or from non-primed synthesis as reported for rheovirus and *E. coli* (see review by Edmonds & Winters, 1976). This assumption, however, fails to fully explain the results obtained for quail since the length of only 3 % of oligo/dT/-bound RNA arising at cleavage remains within the length range of 75–120 bases, corresponding with the usual size of poly/A/ segments (Fig. 4).

An abundance of short oligo/A/ sequences in poly/A/$^+$RNA synthesized in quail at cleavage cannot be ruled out. In sea urchin Duncan & Humphreys (1981) have found at cleavage mRNA very rich in $/A/_{10}$ segments. However, these authors have considered it to be not fully processed pre-mRNA of maternal origin, while in this work we dealt with sequences labelled during cleavage.

Polyadenylation of preexisting RNA, known to occur in 1-cell mouse embryo (Clegg & Pikó, 1982) and in 2-cell sea-urchin embryo (Slater, Slater & Gillespie, 1974; Wilt, 1977), would be the most plausible explanation, even though the presence of stocked maternal RNA in the avian egg has not so far been proved. The heterogeneous pattern of $(A)_n$ tracts rich in short segments (Fig. 5A) may

reflect the early phase of polyadenylation when poly /A/ tails at the 3 ends of the preexisting RNA molecules are just growing.

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