Identification of the major polyadenylylated transcription products and the genes active in their synthesis in a rat insulinoma

(preproinsulin/insulin genes/restriction fragments/nucleic acid hybridization)

JOHN R. DUGUID AND DONALD F. STEINER

Department of Biochemistry, The University of Chicago, Chicago, Illinois 60637

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ABSTRACT Three RNA fractions, with molecular weights of 200,000, 280,000, and 360,000, have been identified as the major polyadenylylated transcription products in a transplantable rat islet cell tumor that synthesizes insulin. These three RNAs share sequence homology as demonstrated by comparisons of both partial and complete RNase T1 digestion products. The 200,000 and 280,000 molecular weight species hybridize primarily with three EcoRI restriction fragments of the rat genome having molecular weights of $1.4 \times 10^6$, $1.6 \times 10^6$, and $6.0 \times 10^6$. The 360,000 molecular weight species hybridizes preferentially with the $6.0 \times 10^6$ molecular weight DNA fragment.

We have previously reported the preparation, from a transplantable rat islet cell tumor, of a mRNA fraction that directs the synthesis of preproinsulin in the wheat germ cell-free system (1). The specific translation activity of this fraction was slightly greater than that of rabbit globin mRNA purified by a similar procedure. The insulin mRNA migrated as a single symmetrical band when electrophoresed on polyacrylamide gels in the presence of formamide with an observed molecular weight of approximately 200,000. Fragments of the mRNAs coding for rat preproinsulin I and II have recently been cloned and sequenced by Ullrich et al. (2).

Our current studies have centered on the identification and purification of the genomic DNA sequence involved in the expression of insulin mRNA. We have found three EcoRI restriction fragments of the rat genome that hybridize to our insulin mRNA probe, with molecular weights of $1.4 \times 10^6$, $1.6 \times 10^6$, and $6.0 \times 10^6$.

In the process of further purifying and characterizing the insulin mRNA fraction we have identified two larger polyadenylylated RNAs that share sequence homology with portions of insulin mRNA. We present here a characterization of these RNAs by analysis of RNase T1 digestion products and by comparison of the genomic DNA sequences to which they are complementary.

METHODS AND MATERIALS

Tumors. The transplantable insulinoma used in these studies was obtained from W. Chick (3) and maintained by serial subcutaneous passage in our laboratory by A. Labrecque. The tumors used in this study weighed 60–150 mg and were stored at $-70^\circ$.

Islet Cell RNA. RNA fractions used in this study were purified by oligo(dT)-cellulose chromatography and sucrose density gradient centrifugation before iodination as described (1).

Electrophoresis. Iodinated RNAs were electrophoresed on polyacrylamide gels in formamide (4) with rat tRNA and yeast tRNA as molecular weight standards (1). Iodinated RNA was located by autoradiography at $-70^\circ$ using Kodak XR-5 film and DuPont Lightning Plus intensifying screens; molecular weight standards were located by ethidium bromide fluorescence. Iodinated RNA was eluted from homogenized gels by shaking overnight at $37^\circ$ in 10% phenol/1% sodium dodecyl sulfate/10 mM Tris/1 mM EDTA, pH 7.5, in the presence of carrier tRNA. The eluted RNA was phenol extracted, ethanol precipitated, and purified by oligo(dT)-cellulose chromatography (1) before further use.

DNA was electrophoresed on 0.7% agarose gels (Bio-Rad) as described by Helling et al. (5) and transferred to cellulose nitrate sheets (Millipore HAWP) by the Southern technique (6). The immobilized DNA was hybridized, to a C$_t$ of approximately $5 \times 10^{-4}$ mol sec/liter, with RNAs obtained from the rat islet cell tumor at $72^\circ$ in 0.9 M sodium chloride/0.09 M sodium citrate/100 $\mu$g of yeast tRNA per ml/1% sodium dodecyl sulfate, pH 7.0 (the RNAs were Millipore-filtered prior to addition of the sodium dodecyl sulfate). The cellulose nitrate filters were then washed with 0.9 M sodium chloride/0.08 M sodium citrate extensively at $70^\circ$ and then treated with ribonuclease A (Worthington) (20 $\mu$g/ml in 0.45 M sodium chloride/0.045 M sodium citrate) for 30 min at room temperature before autoradiography as described above.

Two-Dimensional RNA Fingerprints. Iodinated RNAs were digested in 10 mM Tris (pH 7.5) at $37^\circ$ for 1 hr with RNase T1 (Boehringer) at a weight ratio to carrier tRNA of 1:30. Electrophoresis and homochromatography were performed as described by Brownlee et al. (7) using cellulose acetate strips from Schleicher and Schnell and DEAE-cellulose thin-layer plates from Analtech (Newark, NJ).
of the isolated bands with the G-specific RNase, T1. The profiles obtained from the three RNAs were similar (Fig. 2), indicating a possible shared sequence homology. The discriminating power of this technique is also shown in Fig. 2 by the partial digests of duck globin mRNA and rat 18S rRNA presented.

Components I, II, and III were also digested to completion with RNase T1 and fingerprinted by using the method of Brownlee et al. (7). The autoradiographs (Fig. 3) demonstrated sequence homologies, as well as significant differences, among the three RNAs. The resolved oligonucleotides derived from component I (Fig. 3A) were numbered, and these numbers were assigned to spots at equivalent positions in the fingerprints of components II and III. The map of component II appears to have all the spots present in that of component I with at least two additional spots, labeled "+" in Fig. 3B. The map of component III contains many spots not present in that of component II, indicated by "+" in Fig. 3C. At least one spot present in the maps of components I and II appears to be absent in the map of III; its equivalent position is marked with "-".

Hybridization of Polyadenylated Islet Cell RNAs to Restriction Fragments of the Rat Genome. High molecular weight DNA was prepared from rat spleen, cleaved with EcoRI restriction endonuclease, and fractionated by actinomycin D-cesium chloride density gradient centrifugation (unpublished data). Gradient fractions that hybridized with component I were electrophoresed on agarose gels and transferred to cellulose nitrate sheets by the Southern technique (6). The immobilized DNA was then hybridized with component I. Autoradiographs of the hybridized sheets demonstrated the presence of three bands Fig. 4B. Hybridization of identical sheets with components II and III were performed and the autoradiographs are presented in Fig. 4C and D.

The molecular weights of the hybridizing restriction fragments were determined by using bacteriophage λ EcoRI restriction fragments as internal standards (Fig. 4A), yielding molecular weights for the restriction fragments complementary to the islet cell RNAs of 1.4 × 10^6, 1.6 × 10^6, and 6.0 × 10^6.

The DNA fragments complementary to the islet cell RNAs were purified by RPC-5 chromatography (unpublished data) and used in an experiment similar to that described above. Hybridization with component I revealed the presence of three positive bands with the molecular weights given above (Fig. 5, lane A). Component II hybridized with the same three bands (Fig. 5, lane B); component III hybridized preferentially with the 6.0 × 10^6 dalton fragment (Fig. 5, lane C).

The possibility exists that the three RNAs hybridize to unrelated restriction fragments of the same molecular weight. In the case of the 6.0 × 10^6 dalton DNA fragment, this possibility was ruled out by comparing digests generated with a series of restriction endonucleases: EcoRI, Hpa I, BamHI, Pst I, and Hae II. The digests were electrophoresed on agarose gels, transferred to cellulose nitrate sheets, and hybridized with the three islet cell RNAs. The results of this experiment (Fig. 6) demonstrate that the pattern obtained after hybridization with the three RNAs was identical, indicating that all three hybridize to the same EcoRI restriction fragment.

FIG. 1. Electrophoresis of polyadenylated islet cell RNAs on formamide/polyacrylamide gels. (A) Autoradiography of iodinated islet cell RNAs electrophoresed on 4.1% polyacrylamide gels (4) in a 1-mm-thick 17-cm-long slab gel at 200 V for 3.5 hr. (B) Rat rRNA and yeast tRNA were included as standards and used to construct the molecular weight calibration curve (1). (Inset) Isolated components I, II, and III were electrophoresed as described above, without (lanes C) or with (lanes F) prior treatment with formaldehyde (1). In this experiment component I was split. In this and following figures, the direction of electrophoresis was downward.
FIG. 2. Electrophoresis of partial RNase T1 digests of isolated islet cell RNAs. The iodinated RNAs were dissolved in 80 μl of 40 mM tricine, pH 7.5, with 60 μg of yeast tRNA, heated to 65° for 5 min, made 0.2 M in NaCl, and cooled to 0° at a rate of 0.5°/min. RNase T1 (3.5 ng) was added and the reaction was stopped after 5 min at 0° by phenol extraction. Samples were precipitated with ethanol and electrophoresed on a 1-mm 8.2% formamide/polyacrylamide slab gel (4) at 200 V for 4 hr. The autoradiographs represent digests as follows: lane A, component I; lane B, component II; lane C, component III; lane D, duck globin mRNA; lane E, 18S rat rRNA. 18S rat rRNA and duck globin mRNA were purified and iodinated by standard techniques.

DISCUSSION

In our previous study (1) we did not observe components II and III, although the sensitivity and resolving power of the techniques used should have been sufficient for their detection. A possible reason may be that the tumors used in the previous study were larger (1–2 g) and contained areas of necrosis and cystic degeneration. The tumors used in this study weighed less than 150 mg each and were neither necrotic nor cystic.

Components II and III were first observed in fractions that were active in the synthesis of preproinsulin (Fig. 1), pooled from a sedimentation velocity sucrose gradient. We have found that components II and III have a somewhat greater sedimentation velocity than component I. By analyzing fractions across the sucrose gradient it was found that components II and III are approximately 30–50% as abundant as component I (data not shown).

In similar studies with globin mRNA from both rabbit and duck, isolated and iodinated by the same techniques used in this study, we have never observed evidence of aggregation. The stability of components II and III to formaldehyde treatment, as well as their characterization presented here, indicates that these RNAs are not aggregates of component I.

The partial RNase T1 digests presented in Fig. 2 indicate that the three RNAs share both primary and secondary structural features. The two-dimensional maps of complete RNase T1 digests of the three RNAs confirm this finding. The maps of components I and II are superimposable and the only observed difference was the addition of two spots in the map of component II (see Fig. 3B). The increased complexity of the fingerprint of component III is expected of a molecule nearly twice the size of component I. In addition, the preparation of component III is contaminated with a highly complex population of RNAs, causing a uniform background in the region of the map where higher molecular weight oligonucleotides would be found. Although these problems complicate the interpretation of the fingerprint of component III, shown in Fig. 3C, it is apparent that there are sequences present in component III not present in the two smaller RNAs, as expected from its larger molecular weight. It is notable, however, that there appear to be spots that are present in the smaller RNAs and absent in component III.

We investigated the hybridization of the three islet cell RNAs with restriction fragments of the total rat genome. The criterion used would be expected to allow significant annealing of only the more prominent RNA species present in our preparations of components I, II, and III. The fact that we observed a limited number of DNA fragments complementary to the three preparations indicates that each contains a limited number of major species.

We have found that, whereas components I and II hybridize to three different EcoRI-generated restriction fragments of the rat genome, component III hybridizes preferentially with the 6.0 × 10^6 dalton fragment as shown in Figs. 4 and 5. We have observed that the relative degree of hybridization to different bands in the same gel varies somewhat in different experiments; however, the preferential hybridization of component III to the 6.0 × 10^6 dalton fragment was observed in five independent determinations. This observation may be related to our interpretation of the two-dimensional maps of the three RNAs—that is, components I and II may contain sequences not present in component III.

In conclusion, it is likely that component I represents at least one of the mRNAs that direct the synthesis of the two rat insulins (8), on the basis of its cosedimentation with the peak of preproinsulin synthetic activity in sedimentation velocity experiments, its high specific translation activity, and its apparent high purity as demonstrated by two-dimensional maps of its RNase T1 digestion products, and its selective hybridization to three EcoRI fragments of the rat genome. The identity of components II and III is less clear. The simplest interpretation of our results would be that the two larger RNAs represent precursors to component I—i.e., of one or both of the mRNAs of the two rat preproinsulins. Several laboratories have recently reported the characterization of larger precursors of the mRNAs for a number of proteins (9–14). Furthermore, Leder and coworkers have shown that an inserted sequence in the coding region of the mouse β-globin gene is represented in the 15S β-globin mRNA precursor (15, 16). Thus, the existence of larger RNA molecules sharing sequences with translatable insulin
FIG. 3. Two-dimensional fingerprints of complete RNase T1 digests of islet cell RNAs. Purified components I, II, and III were digested to completion and applied to cellulose acetate strips for electrophoresis at 3000 V for 1.5 hr. The oligonucleotides were then transferred to DEAE-cellulose thin-layer plates for homochromatography using homomix C (7). Autoradiographs of the maps of components I, II, and III are presented in A, B, and C, respectively. Electrophoresis was to the left and homochromatography was upward. Doubling the digestion time neither altered the patterns obtained nor decreased the amount of material remaining at the homochromatography origins.

FIG. 4. Identification of EcoRI-cleaved rat DNA fragments complementary to polyadenylated islet cell RNAs. EcoRI-restricted rat spleen DNA was centrifuged on actinomycin D-cesium chloride gradients and fractions were assayed by hybridization with component I or component III with identical results. The positive fractions were prepared for electrophoresis by isopropanol extraction, dialysis, and lyophilization. Samples (4 μg) of each fraction were applied to a 4-mm thick 0.7% agarose gel (5) with 0.3 μg of EcoRI-cleaved bacteriophage λ Claq7 DNA (a gift from Kevin McEntee, Stanford University) for electrophoresis at 60 V for 8 hr. The resolved DNA was transferred to cellulose nitrate filters and prepared for hybridization as described by Southern (6). Autoradiographs of the filters after hybridization with components I, II, and III are presented in B, C, and D, respectively. The position of λ DNA fragments was determined by rehybridizing the filters, after a 0.5-hr incubation with 0.1 M iodoacetate, with synthetic [3H]RNA complementary to λ and autoradiography as described by Southern (6). The positions of the λ-EcoRI fragments B, C, D, and E in the experiment shown in B is presented in A photographically superimposed in the autoradiograph from B. These data were used to determine the molecular weights presented in the text (5).
mRNA presents the possibility that the insulin genes may have a similar structural organization.

The relatively large abundance of components II and III contrasts with the very low abundance of the 280,000- and 600,000-dalton precursors to globin mRNA (17). However, it may be speculated that tumors such as the malignant x-ray-induced tumors used in this study may have altered mRNA transcription or processing mechanisms, just as many of these tumors have protein precursor-processing deficiencies (17). Whether such defects as ectopic hormone synthesis (18) could result from the translation of improperly processed or aberrant transcription products remains an important consideration for further investigation.

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Fig. 5. Hybridization of polyadenylated islet cell RNAs to purified rat DNA EcoRI restriction fragments. The restriction fragments identified in Fig. 4 were purified by RPC-5 chromatography and used in an experiment analogous to that shown in Fig. 4. The results obtained by hybridizing immobilized DNA with components I, II, and III are presented in lanes A, B, C, and D, respectively.

Fig. 6. Cleavage of the 6.0 × 10⁶ dalton restriction fragment with a series of restriction enzymes. The 6.0 × 10⁶ dalton restriction fragment complementary to islet cell RNA was purified by RPC-5 chromatography. Samples (2 µg) were cleaved with a series of restriction enzymes (EcoRI, Hpa I, BamHI, Ps I, and Hae II) with an internal standard of λ DNA. The digests were electrophoresed on agarose gels for 6 hr (with the exception of the Hae II digests which were electrophoresed for 6 hr) and transferred to cellulose nitrate sheets. The results of hybridization of the sheets with components I, II, and III are presented in A, B, and C, respectively. The molecular weights of the hybridizing restriction fragments, determined by using internal λ standards as described above, are 5.9, 5.9, 0.9, 6.1, and 4.3 × 10⁶ for EcoRI, Hpa I, BamHI, Ps I, and Hae II digests, respectively. Restriction enzymes were obtained from Bethesda Research Laboratories, Rockville, MD, and used as suggested in the supplier's catalog.