STAGE SPECIFIC GENE EXPRESSION PRECEDES MORPHOLOGICAL CHANGES DURING *TRYPANOSOMA CRUZI* METACYCLOGENESIS

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The transformation of epimastigotes to metacyclic trypomastigotes of the *Trypanosoma cruzi* clone Dm 28c has been studied in an in vitro system consisting of artificial triatomine urine supplemented with new born calf serum. The comparison of morphological data with gene expression products, as judged by the proteins synthesized during differentiation, has shown that stage specific gene activation precedes by far the morphological changes of differentiating cells. Immunoprecipitation of differentiating cell antigens with a trypomastigote stage specific antiserum has shown that although the morphological differentiation process takes six days to be completed, epimastigotes start to express the M_r 86 000 and the 78 000 trypomastigote antigens within the first 12 h of induction.

Key words: Chagas' disease; *Trypanosoma cruzi* cloned strain; Gene expression; Cell differentiation

INTRODUCTION

The transformation of epimastigotes into metacyclic trypomastigotes, the natural infective form, also known as metacyclogenesis, is a crucial step in the *Trypanosoma cruzi* life cycle. This process occurs naturally within the triatomine insect vector digestive tract [1,2]. The morphological events which occur during *T. cruzi* differentiation have been extensively studied [3–5] although the physiological basis as well as the molecular aspects of the process remain to be elucidated.

The development of experimental conditions allowing the axenic culture of *T. cruzi*

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Abbreviations: SDS, sodium dodecyl sulphate; NP-40, Nonidet P-40; EDTA, ethylenediamine tetraacetate; PMSF, phenylmethylsulfonyl fluoride; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; TCA, Trichloroacetic acid; TAU, triatomine artificial urine; TAUS, triatomine artificial urine supplemented with 10% newborn calf serum; LIT, liver infusion tryptose medium.

opened the possibility of in vitro differentiation studies, since metacyclic trypomastigotes appear 'spontaneously' in old axenic medium [6]. In addition, several axenic media have been developed allowing the transformation of *T. cruzi* epimastigotes to metacyclic trypomastigotes at 28°C [7–11]. These in vitro differentiating *T. cruzi* systems are important because they render feasible the molecular study of the metacyclogenesis process, which is otherwise difficult since the yield of metacyclic trypomastigotes is very poor when they are obtained directly from the insects [12,13].

We have recently developed a model system allowing in vitro differentiation of *T. cruzi*. In this system, epimastigotes of the cloned *T. cruzi* Dm 28c strain [14] are incubated in a medium simulating reduviid urine supplemented with new born calf serum (Contreras, unpublished results) in such a way that at the end of six days of incubation at 27°C, up to 95% metacyclic trypomastigotes can be obtained. Furthermore, the differentiation is performed without a net increase in the cell population since a stationary phase of growth is simulated.

In this study we have investigated in vitro the transformation of *T. cruzi* epimastigotes to metacyclic trypomastigotes by analysing in vivo translation products, in an attempt to establish the molecular basis for this event. Furthermore, we wished to determine whether morphological modifications were accompanied by a different pattern of in vivo synthesized proteins. Our results show that stage specific gene activation precedes by several days the morphological changes occurring in the epimastigote-trypomastigote transformation of *T. cruzi* Dm 28c clone.

MATERIALS AND METHODS

Cells. The *T. cruzi* Dm 28c clone was isolated from a stock maintained in the Department of Parasitology of the University of Carabobo, Venezuela. The production and characterization of the clone will be published elsewhere ([14], and Contreras et al., in preparation). The clone is maintained by weekly passages through liver infusion tryptose (LIT) medium [7] with a passage through invertebrate and vertebrate hosts every three months.

In vitro differentiation. Parasites from an eight day old culture in LIT medium were centrifuged at 10 000 × g for 15 min at 10°C (Beckman rotor JS 13) and cultured in freshly prepared LIT medium for 48 h at 27°C. Cells were harvested as described above and incubated for 2 h at 27°C with triatomine artificial urine (TAU) (190 mM NaCl, 8 mM phosphate buffer, pH 6.0, 17 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$). Parasites (97–100% epimastigotes) were diluted to a final concentration of 10^6 cells ml^{-1} with TAU supplemented with 10% new born calf serum (Flow Labs) (TAUS medium) and incubated for different times at 27°C in 10 ml aliquots in milk dilution flasks (surface area 25 cm^2), without agitation. 1 ml triplicates of cells in LIT and in TAUS media were placed in assay tubes of 16 × 150 mm (Kimax) and incubated at 27°C for times varying according to the experimental schedule. The in vitro differen-

tiation was monitored by counting cells in a hematocytometer and by differential counting in Giemsa stained smears.

In vivo labelling of parasites with [^{35}S]methionine. 1×10^7 parasites incubated in TAUS for different times were centrifuged at $12\,000 \times g$ for 3 min at room temperature (Eppendorf microfuge) and washed once in TAU. Alternatively, cells were chromatographed in DEAE-cellulose columns [15,16] in order to obtain a 100% metacyclic trypomastigote population.

Cells washed in TAU were sedimented as described above and incubated for 120 min in one ml of Hanks' solution supplemented with 20 mM Hepes pH 7.4 and 100 µCi [^{35}S]methionine (1045 Ci mmol^{-1}, New England Nuclear), at 27°C. The incorporation of [^{35}S]methionine as hot trichloroacetic acid precipitable material was linear under the experimental conditions employed. Following the 2 h incubation 10 µg cyclohexi- mide (Sigma Chemical Co.) were added and the cells incubated for a further 15 min. Labelled parasites were centrifuged for 3 min at $12\,000 \times g$ at room temperature (Eppendorf microfuge), resuspended in 500 µl of lysis solution (150 mM NaCl, 10 mm Tris-HCl, pH 7.4, 1 mM ethylenediamine tetraacetate (EDTA), 1 mM phenylmethyl- sulfonyl fluoride (PMSF), 2 mM iodoacetamide, 1% Nonidet-40 (NP-40) homogeniz- ed for 3 min at room temperature and centrifuged for 10 min at $12\,000 \times g$ (Eppendorf microfuge). The supernatant was immediately processed for immunopre- cipitation or electrophoresis or alternatively, stored at –70°C for later analysis. The concentration of proteins was determined according to Spector [17].

Preparation of the anti-epimastigote serum. New Zealand rabbits weighing 3 kg were inoculated twice intramuscularly at 8 day intervals with 10^8 epimastigotes with Freund complete adjuvant. Following this, they were inoculated intravenously three times at 48 h intervals with 10^7 live epimastigotes in Hanks' solution. A week after the last inoculation the immune response was assayed by indirect immunofluorescence [18] with the homologous antigen obtaining a title of 1:640. The anti-epimastigote serum was kept in aliquots at –70°C.

Preparation of trypomastigote stage specific antiserum. 10^8 *T. cruzi* Dm 28c trypo- mastigotes obtained from in vitro infected fibroblasts were fixed with 0.25% glutaral- dehyde, resuspended in 2 ml of Freund complete adjuvant and injected subcutaneously in the scruff of the neck of New Zealand rabbits. Metacyclic trypo- mastigotes (2×10^8), induced in the semi-defined TAUS differentiating medium, were incubated at 37°C for 30 min with 5 ml of the globulin fraction of the anti-epimastigote serum and sonicated at 4°C in the presence of 1 mM PMSF. To the effluent of a protein A-Sepharose (Pharmacia) affinity column of this material we have added an equal volume of Freund complete adjuvant and the rabbits have been injected intramuscu- larly 8 days after the first inoculation. A week later, the rabbits were inoculated intravenously 3 times, at 48 h intervals, with 10^7 in vitro induced metacyclics. Six days

after the last inoculation the rabbits were bled and the antiserum obtained was adsorbed 4 times with 3 g of 100% live epimastigotes. A titer of 1:100 has been determined for this antiserum by indirect immunofluorescence against in vitro induced metacyclic trypomastigotes.

Immunoprecipitation. [^{35}S]methionine-labelled parasite lysates were clarified by centrifugation at 12 000 × g for 3 min at room temperature. To a 20 μl aliquot of clarified lysates we added 5 μl of 70 mg ml⁻¹ bovine serum albumin, pH 8.0, 2 mM PMSF and 10 μl of a normal rabbit serum. The non-specific immunoprecipitation was carried out twice for 30 min at room temperature in a final volume of 100 μl in immunoprecipitation buffer (10 mM Tris-HCl, pH 8.3, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate). To this we added 20 μl of 10% formalin fixed *Staphylococcus aureus* (EcSorb, EY Labs) [19], incubated for a further 10 min at room temperature and finally centrifuged at 12 000 × g for 5 min (Eppendorf microfuge). After the second round of non-specific immunoprecipitation no polypeptides could be detected by polyacrylamide gel electrophoresis. The supernatants were incubated with 20 μl of the desired antiserum at 4°C overnight and the specific immune-complexes were collected with *S. aureus* cells as described above. The pellets were washed five times with 1 ml of 50 mM Tris-HCl, pH 8.7, 300 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% NP-40. The polypeptides were dissociated by boiling the immunoprecipitates for 3 min in 20 μl of electrophoresis sample buffer [20].

Polyacrylamide gel electrophoresis and fluorography. This was performed as described for one dimensional gels [20] using a 5–15 or 10–15% linear sodium dodecylsulfate SDS-polyacrylamide gradient. Two dimensional polyacrylamide gel electrophoresis was performed as described by O'Farrel [21]. The second dimension consisted of a uniform 13% SDS-polyacrylamide gel [20]. One and two-dimensional polyacrylamide gels were fixed overnight with 10% acetic acid, 30% methanol and treated for fluorography with Enhance (New England Nuclear Inc.). The dried gels were exposed at −70°C to X-ray films (Sakura, Japan) using a Kodak X-O-Matic regular intensifying screen.

RESULTS

In vitro metacyclogenesis of T. cruzi. The incubation of LIT medium epimastigotes of the cloned *T. cruzi* Dm 28c strain in TAUS results in the gradual appearance of metacyclic trypomastigotes in a period of six days (Fig. 1). During the first 48 h of incubation the formation of cell aggregates was observed (Fig. 1). An important feature of this differentiation medium is the fact that, under the experimental conditions employed, the process of cell transformation was not accompanied by a net increase in the cell population. After six days of cell incubation in the differentiating medium, titers as high as 85 ± 5% metacyclic trypomastigotes are obtained (Table I).
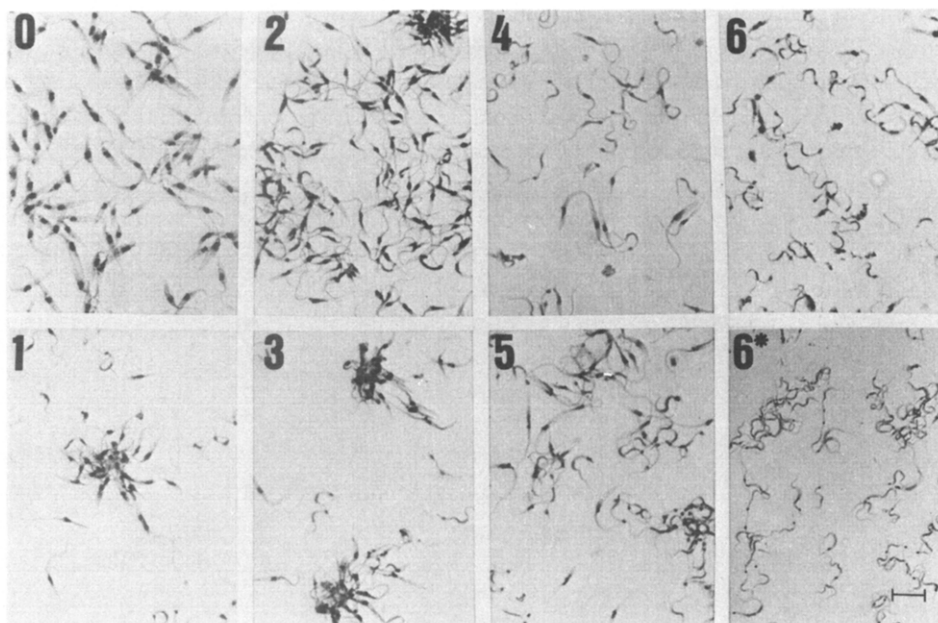
Fig. 1. Smears from *T. cruzi* Dm 28c cells on different days of in vitro differentiation. The numbers refer to the distinct days. 6* are cells from day 6 that have been chromatographed through DEAE-cellulose. The bar at the bottom represents 10 μm.

TABLE I

Specific activity and percentage of metacyclic trypomastigotes during the differentiation of *T. cruzi* Dm 28c epimastigotes to trypomastigotes.

Day of differentiation	% of metacyclic trypomastigotes [a]	cpm μg^{-1} of protein
0	0	39 144
1	8 ± 2	20 670
2	15 ± 9	20 979
3	35 ± 5	20 258
4	63 ± 7	19 888
5	73 ± 8	23 694
6	85 ± 5	22 307
6*, [b]	100	5 200

[a] Differential cell counting was performed either in Neubauer chambers or in permanent smears stained with Giemsa.

[b] Day 6* are day 6 cells that have been chromatographed on DEAE-cellulose prior to labelling.

Incubation for longer times did not increase significantly the amount of metacyclic trypomastigotes (not shown). Nevertheless, purified metacyclic trypomastigotes could be obtained by cell column chromatography in DEAE-cellulose (Fig. 1, 6*). These column-purified metacyclics display biological properties similar to those isolated from triatomine excreta (Contreras et al., in preparation).

The specific activity of cells pulse-labelled for 2 h with [^{35}S]methionine decreased 50% from day 0 to day 1, but then remained constant until day 6, despite the increasing percentage of trypomastigotes (Table I). A marked difference in specific activity is observed for the purified metacyclic trypomastigotes population in relation to the original epimastigotes. This could either reflect metabolic particularities of each differentiation stage or a reduction of metabolic activity due to the column purification step.

Analysis of in vivo synthesized proteins. We have analysed, by two dimensional polyacrylamide gel electrophoresis, the proteins synthesized during the in vitro differentiation of *T. cruzi* Dm 28c epimastigotes to metacyclic trypomastigotes (Fig. 2). Fig. 2A shows the protein profile of LIT medium epimastigotes that were used for the in vitro differentiation in TAUS medium. The comparison of this protein profile with the protein profile displayed by cells incubated for 1 day in TAUS (Fig. 2B) shows important changes, represented by the disappearance of some polypeptides (1 and 14, Fig. 2A), the lowering in intensity of others (e.g. group 6 in Fig. 2A) and the appearance of several distinct groups of polypeptides (2,3,7,8,9,10 and 11, Fig. 2B). When this last profile is compared with those depicted by cells from day 2 (Fig. 2C), day 4 (Fig. 2D) and day 6 (Fig. 2E), it is clearly seen that the protein profiles are remarkably conserved, despite the differences in the amount of metacyclic trypomastigotes present (Table I). Indeed, the protein profile displayed by cells from day 1 of differentiation (Fig. 2B) resembles the protein profile presented by purified metacyclic trypomastigotes (Fig. 2F). Most of the polypeptides are conserved with the exception of those of groups 10 and 11 that disappear and two new polypeptides (12 and 13, Fig. 2F) that are synthesized by purified metacyclic trypomastigotes. It is interesting to note that culture epimastigotes synthesize mainly acidic polypeptides while differentiating cells present a broad spectrum of synthesized polypeptides (Fig. 2). These results point to the fact that under the conditions employed, cells which are still morphologically classified as epimastigotes may display a protein biosynthesis pattern characteristic of metacyclic trypomastigotes.

In order to get further insight into the transformation of epimastigotes to trypomastigotes and to investigate whether epimastigotes might be expressing trypomastigote stage specific proteins and not only proteins common to both stages, we immunoprecipitated the in vivo labelled products from each day of differentiation with *T. cruzi* stage specific antisera. Fig. 3, lanes A to H, shows the labelled polypeptides from day 0 to day 6, respectively, immunoprecipitated with an antisera raised against epimastigotes. It is observed that the transition from epimastigotes (Fig. 3 lane A) to one day
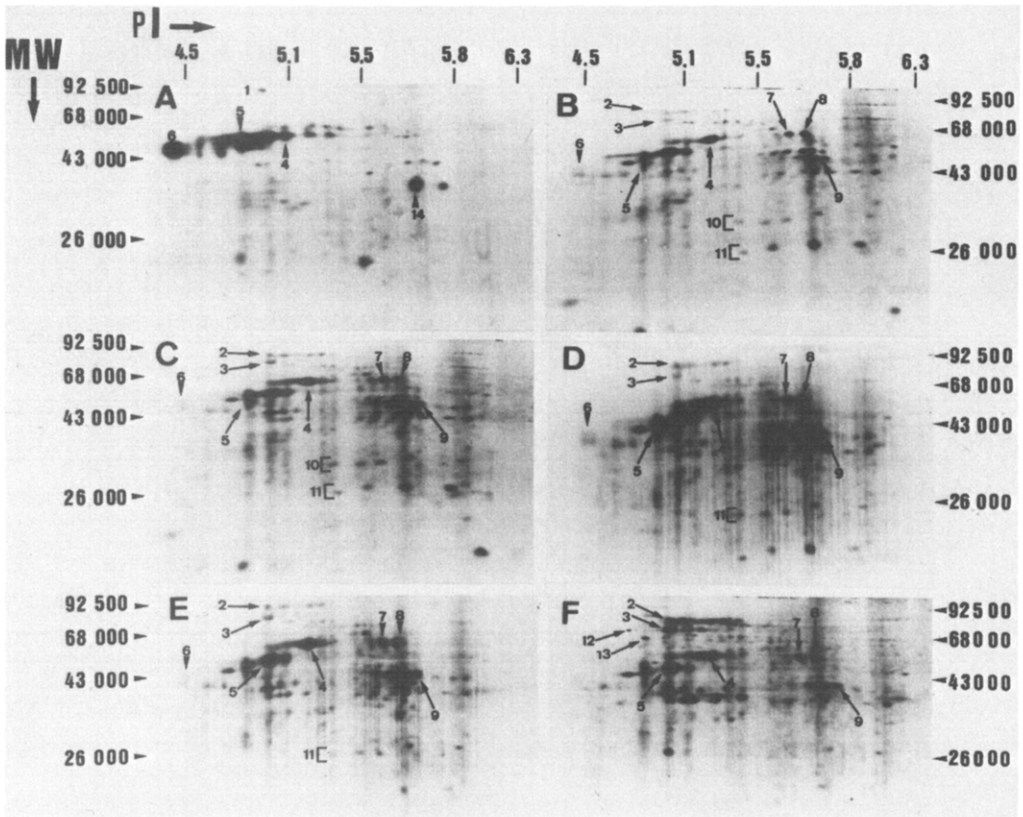
Fig. 2. Two-dimensional polyacrylamide gel electrophoresis of the in vivo translation products from *T. cruzi* Dm 28c cells. A to E refer, respectively, to cells from days 0, 1, 2, 4 and 6 of in vitro metacyclogenesis. F refers to cells from day 6 that have been chromatographed on DEAE-cellulose prior to labelling with [^{35}S]methionine. First dimension is an isoelectric focusing and second dimension is a denaturing 13% SDS-polyacrylamide gel electrophoresis. Each gel was loaded with 10^6 cpm. The numbers at the horizontal axis refer to the pH gradient and at the vertical axis to ^{14}C-molecular weight markers run in the same gel.

differentiating cells (Fig. 3 lane B) is accompanied by the disappearance of polypeptides of M_r 75 000, 41 000 and 27 000 and that this last profile is maintained up to day 6 (lane H). Furthermore, the conservation of the other immunoprecipitated polypeptides during the transformation of epimastigotes to trypomastigotes indicates that common antigens are shared by both differentiation stages.

Immunoprecipitation with a trypomastigote-specific antisera pre-adsorbed with epimastigotes (Fig. 3 lanes I to P) shows that the transition of epimastigotes to one day differentiating cells corresponds to the appearance in the latter of two bands of M_r 78 000 and 86 000. This immunoprecipitation profile is maintained during the entire differentiation process (Fig. 3 lanes K to O) and within the purified metacyclic trypomastigote population (Fig. 3 lane P), despite the different amounts of metacyclic
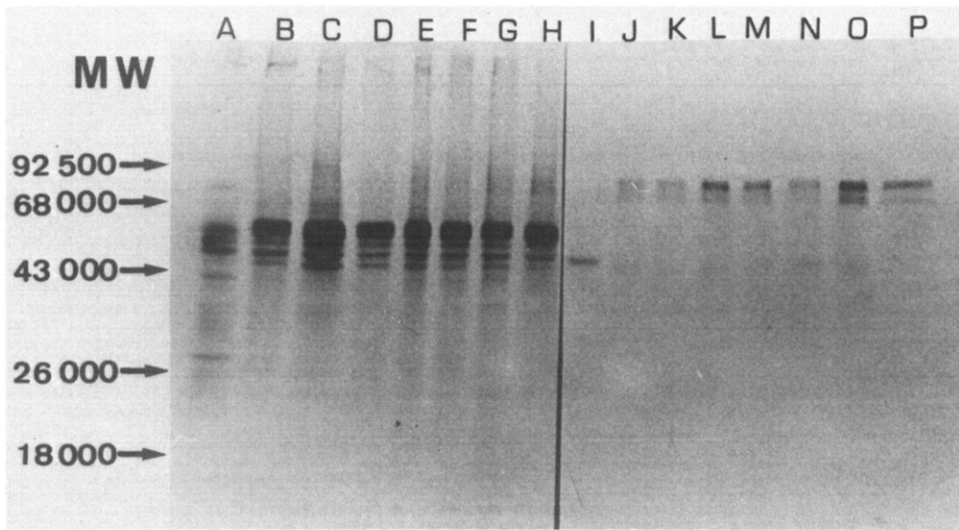
Fig. 3. 5–15% linear gradient SDS-polyacrylamide gel electrophoresis of the polypeptides immunoprecipitated with *T. cruzi* stage specific antisera during in vitro metacyclogenesis. Lanes A to H refer to cells from day 0 to day 6*, respectively, immunoprecipitated with epimastigote antiserum. Lanes I to P show the polypeptides from cells day 0 to day 6* immunoprecipitated with trypomastigote stage specific antiserum. The position of ^{14}C-molecular weight markers of 92 500, 68 000, 43 000, 26 000, 18 000 is shown at the left of the figure.

trypomastigotes. The immunoprecipitation of a single M_r 52 000 polypeptide from epimastigote cells (Fig. 3 lane I) by the trypomastigote stage specific antisera could be explained by the fact that this antisera has been pre-adsorbed with epimastigote living cells. Hence the M_r 52 000 polypeptide could be an internal rather than a surface antigen. The results confirm our previous observation that the main modifications in the gene expression products occur between the first 24 h in our in vitro differentiation conditions. Furthermore, they indicate that, in *T. cruzi*, the differentiation in terms of gene expression products precedes by far the morphological differentiation.

Analysis of the proteins synthesized during the first 24 h of differentiation. Cells have been incubated in TAU for 2 h and in TAUS for 6, 12, 18 and 24 h. Fig. 4A shows the protein profile displayed by cells incubated for 2 h in TAU before labelling (0 h of differentiation). Comparison with Fig. 2A shows that this incubation condition triggered the synthesis of polypeptides 7 and 8. The incubation of the cells for 12 h in the differentiation medium resulted in a more complex protein profile, with the appearance of polypeptide groups 2 and 3 and the reduction of polypeptide 6 (Fig. 4B). At the end of 24 h of incubation (Fig. 4C) the protein profile is identical to that previously observed (Fig. 2B).

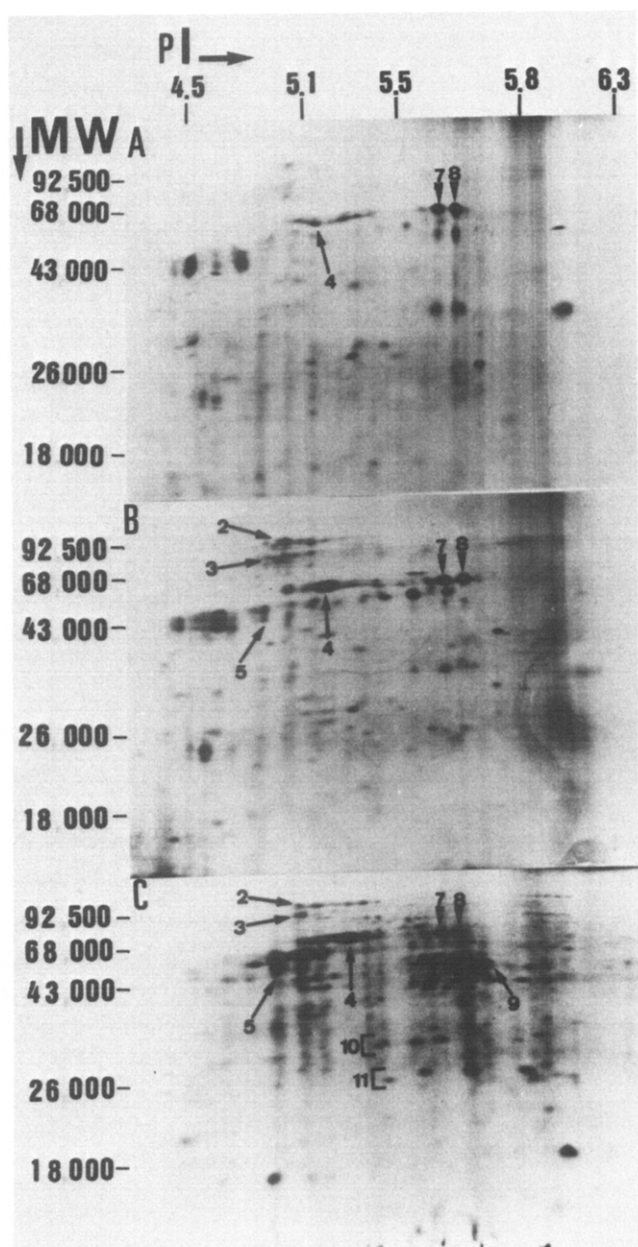The immunoprecipitation of the proteins synthesized during the first 24 h of

Fig. 4. Two dimensional polyacrylamide gel electrophoresis of the [^{35}S]methionine in vivo translation products from *T. cruzi* Dm 28c cells incubated in TAUS medium during: (A) zero h (control), (B) 12 h, and (C) 24 h. First dimension is an isoelectric focusing and second dimension is a uniform 13% SDS-polyacrylamide gel electrophoresis. The position of ^{14}C-molecular weight markers run together in the gels is shown in the left side of the figure.
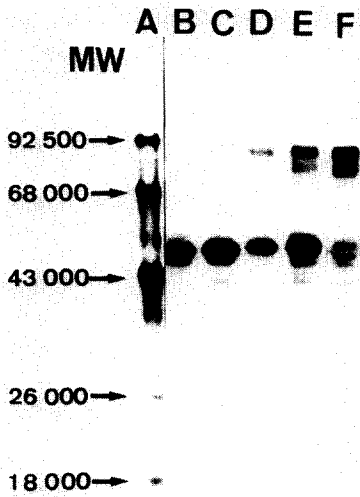
Fig. 5. 10–15% linear gradient SDS-polyacrylamide gel electrophoresis of the polypeptides immunoprecipi-tated with trypomastigote stage specific antiserum from *T. cruzi* Dm 28c differentiating in TAUS medium for (B) 0 h, (C) 6 h, (D) 12 h, (E) 18 h, (F) 24 h. Lane (A) shows ^{14}C-molecular weight markers as in the legend to Fig. 3. Immunoprecipitations were carried out using equal amounts of labelled antigens.

differentiation (Fig. 5), has shown that after 12 h the M_r 78 000 and the 86 000 antigens are already immunoprecipitated by the trypomastigote stage specific antisera (Fig. 5 lane D). This excludes the possibility that these polypeptides originated from culture metacyclic trypomastigotes. No morphological changes occur within the first 12 h of cell incubation in the differentiating medium, thus indicating that stage specific gene expression precedes the morphologica transformation within *T. cruzi* Dm 28c under the experimental conditions employed.

DISCUSSION

Our results characterize some of the molecular events involved in the in vitro transformation of *T. cruzi* epimastigotes to infective metacyclic trypomastigotes. In addition, they point to the fact that cells which are morphologically indistinguishable from epimastigotes are expressing trypomastigote specific gene products after a few hours of triggering the differentiation process.

T. cruzi cells displayed at least three distinct protein profiles during the differenti-ation process. Metacyclic trypomastigotes displayed a more complex pattern than epimastigotes (Fig. 2F and 2A, respectively), in contrast with the specific activity presented by cells from both differentiation stages labelled with [^{35}S]methionine. Cells exposed to the differentiating medium from one to six days (Figs. 2B to 2E) displayed an identical protein profile, despite the differences in the percentage of metacyclic

trypomastigotes. These profiles resemble that of purified metacyclic trypomastigotes with the exception of the group of polypeptides of M_r 34 000 and 27 000 (group 10 and 11, Fig. 2) which could be characteristic of a transition stage since they are not present in epimastigotes under the labelling conditions employed and disappear within the purified metacyclic trypomastigote population.

We can not rule out the possibility that the protein profile presented by the epimastigotes reflects a particular physiological state rather than being typical of this differentiation stage. The metabolic labelling condition as well as the differentiating system consist of poor media. This condition might trigger and synchronize the expression of trypomastigote stage specific gene products. Corroborating this idea, we would like to recall that different stresses induce the appearance of metacyclic trypomastigotes [6,9,22,23] and that these cells are naturally found in a poor medium, the triatomine excreta [24,25].

The immunoprecipitation of metabolically labelled products with stage specific antisera has confirmed that differentiating epimastigotes express trypomastigote stage specific antigens (Fig. 3). The expression of the M_r 78 000 and 86 0000 trypomastigote stage specific antigens is a primary event during the transformation process since they appear within the first 12 h of differentiation (Fig. 5C). These polypeptides are very likely to be surface antigens since polypeptides displaying an identical molecular weight and isoelectric point are immunoprecipitated by this antisera from iodine labelled metacyclic trypomastigotes (manuscript in preparation). As yet, we do not know to which extent these trypomastigote antigens correlate with the proteins described by other groups [26–31] since discrepancies may exist due to the experimental conditions employed [32].

The immunoprecipitation of the synthesized proteins with the epimastigote antisera corroborates the results concerning the timing of gene switching during differentiation. Surprisingly, this antisera recognized few proteins in both differentiation stages, suggesting that this phenomena could be due to the fact that we are analysing proteins synthesized in a short period of time by cells incubated in a nutritionally poor medium.

Evidence for stage specific gene expression preceding the morphological changes during *T. cruzi* differentiation has been presented by Sher et al. [22]. They demonstrated that epimastigotes incubated for 6 h under differentiating conditions displayed resistance to complement lysis, a property characteristic of metacyclic trypomastigote forms [33]. On the other hand, a close relationship has been described among gene expression products and morphological changes during *Leishmania* differentiation [34]. These distinct behaviors could be intrinsic to the cell systems employed.

As yet, it is premature to assume that the in vitro differentiation system we have developed mimicks exactly the epimastigote to trypomastigote transformation that occurs within the invertebrate host. Nevertheless, the in vitro induced *T. cruzi* Dm 28c metacyclic trypomastigotes can not be distinguished from metacyclic trypomastigotes obtained from the excreta of infected triatomines in terms of their biological properties

(Contreras et al., in preparation). This suggests that these in vitro induced metacyclic trypomastigotes, due to their easy preparation, might represent a good source of bona fide trypomastigote antigens and respective messenger RNAs.

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