

## Posttranscriptional Control of Tyrosine Aminotransferase Synthesis by Insulin

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**Abstract.** Adrenal steroid hormones induce the synthesis of tyrosine aminotransferase (TAT) in HTC cells, an established line of rat hepatoma cells in tissue culture. The addition of insulin to cells previously induced in a serum-free medium results in a rapid two- to threefold further increase in the rate of synthesis of TAT and a small increase in total amino acid incorporation. These changes do not require concomitant RNA synthesis, suggesting that insulin acts at a step in protein synthesis beyond that of gene transcription. Although the effects of insulin on HTC cells are similar to those caused by dialyzed bovine serum, evidence is presented that insulin and serum affect different aspects of TAT synthesis. Unlike the glucocorticoids, insulin does not cause the accumulation of TAT mRNA, nor the sustained induction of TAT. The continued presence of the inducing steroid is required to permit maximal expression of the insulin effect.

**Introduction.** Adrenal steroid hormones induce an increased synthesis of tyrosine aminotransferase (TAT) in HTC cells, an established line of rat hepatoma cells in tissue culture.<sup>1,2</sup> Although the induction process requires concomitant RNA synthesis, several lines of evidence suggest that the hormone acts at a step in protein synthesis beyond that of gene transcription.<sup>2</sup>

Previously we have reported that the addition of dialyzed bovine serum to HTC cells, induced in a chemically defined, serum-free medium, results in a rapid two- to threefold further increase in the rate of TAT synthesis.<sup>3</sup> This increase is largely independent of concomitant RNA synthesis, suggesting that serum acts at a posttranscriptional step in enzyme synthesis. In addition, serum enhances over-all protein synthesis by about 40%, and causes a shift in ribosome distribution toward polysomal aggregates.<sup>3</sup> These processes were somewhat more dependent on continued RNA synthesis.

Since insulin has been reported to replace the requirement for serum in some cultured animal cells,<sup>4-6</sup> and to induce the synthesis of TAT in rat liver,<sup>7</sup> perfused rat liver,<sup>8</sup> and fetal rat liver in organ culture,<sup>9</sup> we have investigated the effect of insulin on TAT synthesis in HTC cells. We have observed that insulin, like serum, enhances the synthesis of TAT in HTC cells previously induced in a serum-free medium, by a mechanism which does not require concomitant RNA synthesis. Evidence is presented, however, that insulin and serum influence different biochemical reactions.

**Materials and Methods.** HTC cells were grown in suspension cultures as described previously.<sup>3</sup> Experimental incubations were performed at cell concentrations of 1 to  $1.2 \times 10^6$  cells/ml in serum-free media, either medium S-77 (Grand Island Biological Co.) modified to contain 0.5 gm/liter  $\text{NaHCO}_3$ , 0.05 M Tricine (Calbiochem), and 2 mM glutamine, and designated induction medium, or similarly modified Eagle's minimal essential medium (Microbiological Associates), designated MEM-T. Similar results were obtained with both media.

Bovine serum was obtained from Grand Island Biological Co. Actinomycin D and dexamethasone phosphate were gifts of Merck and Co. Cycloheximide was a gift of the Upjohn Co. Porcine crystalline zinc insulin (Lilly), containing less than 0.03% (w/w) glucagon, was a gift from Dr. Jesse Roth, National Institute of Arthritis and Metabolic Diseases, and Dr. W. Bromer of Eli Lilly and Co.  $^{14}\text{C}$ -l-amino acid mixture (spec. act. 75-410 mCi/mM) was obtained from New England Nuclear.

Experimental manipulations were performed as described in detail previously,<sup>3</sup> and in the legends to the figures.

**Results.** The incubation of HTC cells in a chemically defined serum-free medium with  $10^{-6}$  M to  $10^{-5}$  M dexamethasone phosphate results in approximately a fivefold increase in the activity of TAT. After a lag of about  $1\frac{1}{2}$  hr, enzyme activity increases rapidly, reaching a plateau level after about 8 hr. Actinomycin D at a concentration of 0.15  $\mu\text{g}/\text{ml}$  inhibits RNA synthesis by more than 90% under these conditions and completely inhibits the steroidal induction of TAT<sup>10</sup> (also see Fig. 4).

The addition of 0.1 unit/ml (4  $\mu\text{g}/\text{ml}$ ) insulin to cells previously induced in the absence of serum for 16 hr with  $10^{-6}$  M dexamethasone phosphate results in a two- to threefold further increase in TAT activity, which is maximal within 2 hr after insulin addition and which then falls toward the induced plateau level (Fig. 1). Re-addition of the same concentration of insulin at 2 and 4 hr after the initial addition (not shown) prevents this fall but does not cause a further increase in TAT activity.

Concentrations of insulin as low as 0.005 unit/ml (0.2  $\mu\text{g}/\text{ml}$ ) cause a 50% increase in TAT activity within 2 hr, and in general the magnitude of the increase in TAT activity is linearly proportional to the logarithm of the insulin concentration over a range of 0.001 to 0.1 unit/ml. Higher concentrations, up to 1.0 unit/ml, do not cause a greater response.

As seen in Figure 1, addition of 0.15  $\mu\text{g}/\text{ml}$  actinomycin D 20 min prior to insulin does not significantly reduce the latter's effect on the level of TAT activity. Thus, the synthesis of new TAT mRNA is not required, suggesting that insulin acts by enhancing the translation of preexisting TAT mRNA. The addition of this concentration of actinomycin D alone to the induced cells causes a gradual decline in TAT activity rather than the "superinduction" seen at higher concentrations.<sup>2</sup>

The stimulation of TAT activity by insulin is completely inhibited by  $10^{-4}$  M cycloheximide (Fig. 1), which blocks protein synthesis by more than 95%.<sup>10</sup> Therefore, the increase in TAT activity most likely does not represent simply activation of a previously formed inactive precursor. Furthermore, under these experimental conditions, the rate of decay of TAT activity after inhibition of protein synthesis by cycloheximide is an accurate reflection of the actual rate of degradation of TAT as measured by specific immunoprecipitation of labeled

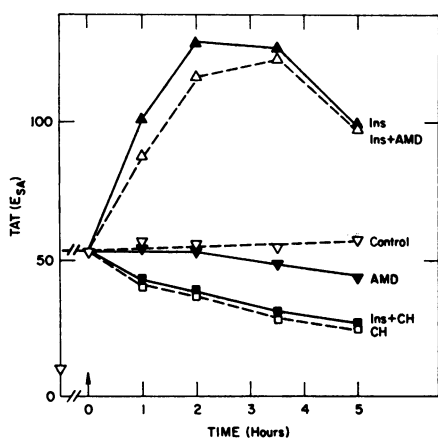


FIG. 1. A culture of HTC cells was incubated in induction medium for 16.5 hr with  $10^{-6}$  M dexamethasone phosphate. After removal of a 2-ml sample for enzyme assay (time 0), 10-ml portions were incubated with the following additions for an additional 5 hr:

—▽—, none (control); —▼—, actinomycin D (AMD) (0.15  $\mu$ g/ml); —▲—, insulin (Ins.) (0.1 unit/ml); —△—, insulin (Ins) (0.1 unit/ml) plus actinomycin D (AMD) (0.15  $\mu$ g/ml); —□—, cycloheximide (CH) ( $10^{-4}$  M); and —■—, cycloheximide (CH) ( $10^{-4}$  M) plus Insulin (Ins) (0.1 unit/ml). Actinomycin D and cycloheximide were added 20 min prior to insulin. 2-ml samples were taken at 1, 3, 3 1/2, and 5 hr and analyzed as described.<sup>3</sup> TAT ( $E_{5A}$ ) (tyrosine aminotransferase spec. act.): m $\mu$ moles product formed/min/mg protein at 37°C.

TAT.<sup>11</sup> Thus, Figure 1 also shows that TAT degradation is not affected by insulin. Taken together, these data suggest that insulin increases the activity of TAT by increasing its rate of synthesis. This conclusion is supported by preliminary experiments, not shown here, in which the rate of TAT synthesis measured by radioimmunoprecipitation techniques<sup>12</sup> was increased by insulin.

The requirement for the glucocorticoid inducer is shown in Figure 2. Removal of dexamethasone phosphate from previously induced cells results in a prompt decay in TAT activity resulting from an almost immediate cessation of TAT synthesis.<sup>2</sup> The addition of insulin to induced cells from which dexamethasone phosphate has been removed still evokes a significant increase in TAT activity; however, the response is markedly reduced relative to that observed in the presence of the steroid hormone. In other experiments not shown here, results similar to the above were obtained when the action of the glucocorticoid hormone was antagonized by 17- $\alpha$ -hydroxyprogesterone.<sup>13</sup> Thus, the glucocorticoid inducer must be present to permit the maximum insulin effect.

In addition to its effect on TAT activity, insulin, like serum, also stimulates

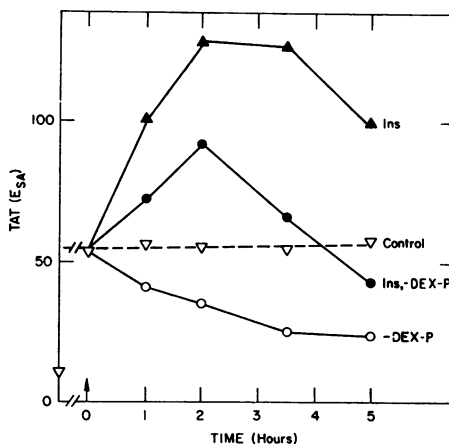


FIG. 2.—A culture of HTC cells was incubated in induction medium for 16.5 hr with  $10^{-6}$  M dexamethasone phosphate (part of the same experiment shown in Fig. 1). After removal of the "time 0" sample, two 10-ml portions were incubated with the following additions (as shown also in Fig. 1):

—▽—, none (control); and —▲—, Insulin (Ins) (0.1 unit/ml). Two other 10-ml portions of the culture were centrifuged at  $1500 \times g$  for 5 min. The cell pellets were washed once with 20 ml fresh induction medium without hormone at 37°C, and resuspended in 10 ml fresh induction medium without dexamethasone phosphate. Insulin was added to one portion of cells.

—○—, minus dexamethasone phosphate (—DEX-P). —●—, minus dexamethasone phosphate (—DEX-P) plus insulin (Ins) (0.1 unit/ml). 2-ml portions were removed at 1, 2, 3 1/2, and 5 hr and analyzed as described.

the incorporation of labeled amino acids into trichloroacetic acid-insoluble material by 20–30%. Approximately half this increase is prevented by actinomycin D and presumably requires the synthesis of new RNA. We do not know whether this increased incorporation results from a large stimulation of the synthesis of a limited number of proteins (of which TAT is representative), or whether the synthesis of all proteins is affected, with TAT synthesis being exceptionally sensitive.

The effect of insulin on the distribution of ribosomes in HTC cells has also been investigated.<sup>10</sup> After induction in a serum-free medium for 12 hr, approximately 50% of the cytoplasmic ribosomes sediment as polysomal aggregates, and the remaining 50% as monomer and dimer units. Ninety minutes after the addition of 0.1 unit/ml insulin, at which time TAT activity has increased by 95%, there is a small increase in the proportion of ribosomes found in polysomal aggregates. In the presence of actinomycin D, however, this shift in ribosome distribution is not observed even though the increase in TAT activity is unaffected.

The effects of 0.1 unit/ml insulin on HTC cells described above—namely, the striking increase in TAT activity which is insensitive to actinomycin D, the modest stimulation of general protein synthesis, and the slight shift in ribosome distribution—are all similar to those observed under the same experimental conditions after addition of 5% bovine serum to the medium. Since insulin can replace the requirement for serum in other experimental systems,<sup>4–6</sup> it seemed possible that insulin in serum was responsible for the effects of serum on HTC cells we have described.<sup>3</sup> This appears unlikely since the concentrations of insulin in bovine serum are only 20–30  $\mu\mu$  units/ml in fasting samples and up to 400 to 500  $\mu\mu$  units/ml after administration of glucose.<sup>14</sup> Thus, the maximal insulin concentration in 5% bovine serum would be 25  $\mu\mu$  units/ml, or 1/4000 the insulin concentration required to give an increase in TAT activity comparable to that obtained with 5% serum.

Furthermore, when maximally effective concentrations of serum (5%) and insulin (0.1 unit/ml) are both added to HTC cells previously induced in a serum-free medium, the increase in TAT activity is approximately the sum of the separate effects of serum and insulin (Fig. 3). The addi-

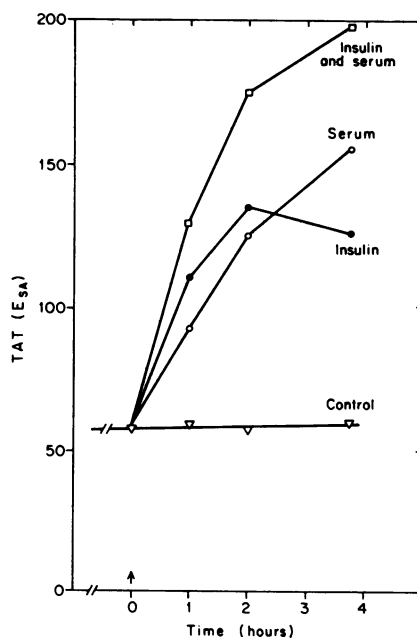


FIG. 3.—A culture of HTC cells was incubated for 15 hr in induction medium with  $10^{-6}$  M dexamethasone phosphate. After removal of a 2-ml sample (time 0) for enzyme assay, four 8-ml portions were incubated for another 4 hr with the following additions:

— $\nabla$ —, none (control); — $\bullet$ —, insulin (0.1 unit/ml); — $\circ$ —, bovine serum (5%); and — $\square$ —, insulin (0.1 unit/ml) plus bovine serum (5%). Two ml samples were taken at 1, 2, and  $3\frac{2}{3}$  hr and analyzed as described previously.

tive effect of insulin and serum on TAT activity is also observed in the presence of actinomycin D.<sup>10</sup> Our results suggest that serum and insulin both affect TAT synthesis at a step beyond gene transcription, but that they must somehow affect different processes.

The continued presence of the glucocorticoid inducer is required for the maximal stimulation of TAT by insulin, as it is for serum. The relation of the mechanisms by which insulin and dexamethasone phosphate affect TAT activity was explored in the following experiments. When HTC cells are incubated with  $10^{-6}$  M dexamethasone phosphate, TAT activity begins to increase after a lag of approximately  $1\frac{1}{2}$  hr and reaches a plateau level after 8 to 12 hr incubation at about five times the basal TAT activity. Actinomycin D, 0.15  $\mu\text{g}/\text{ml}$ , completely prevents the induction of TAT (Fig. 4). In contrast, when HTC cells are incubated with 0.1 unit/ml insulin there is an immediate increase in TAT activity reaching a maximum within about 2 hr at about twice the basal activity, and then declining toward the basal activity level. The increase in TAT activity evoked by insulin is not significantly inhibited by actinomycin D (Fig. 4). The time course, magnitude, and insensitivity to actinomycin D of this effect of insulin on uninduced cells is similar to that seen in previously induced cells (Fig. 1). The simultaneous addition of insulin and dexamethasone phosphate to uninduced HTC cells results in a composite effect on TAT synthesis; that is, an early increase in TAT (insulin effect), followed by a sustained induction of TAT to the same plateau level as seen with dexamethasone phosphate alone (dexamethasone effect).

Steroids are thought to induce TAT by some mechanism involving the accumulation of TAT-specific mRNA.<sup>2,15</sup> Furthermore, dexamethasone phosphate causes the accumulation of this TAT-specific mRNA even in the absence of protein synthesis.<sup>16</sup> Thus, when HTC cells are incubated with dexamethasone phosphate and cycloheximide for 90 min, and then washed free of the inducer and the inhibitor, there is an immediate increase in TAT activity without a lag,

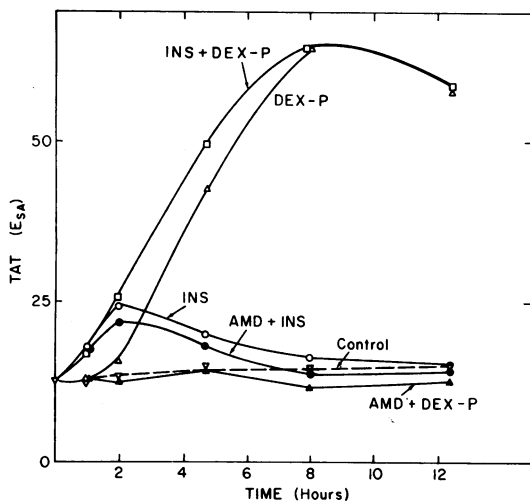


FIG. 4.—Six 12-ml portions of HTC cells in modified Eagle's minimal essential medium were incubated with the following additions:

—▽—, none (control); —△—, dexamethasone phosphate (DexP) ( $10^{-6}$  M); —▲—, actinomycin D (AMD) (0.15  $\mu\text{g}/\text{ml}$ ) plus dexamethasone phosphate (DEX-P) ( $10^{-6}$  M); —○—, insulin (Ins) (0.1 unit/ml); —●—, actinomycin D (AMD) (0.15  $\mu\text{g}/\text{ml}$ ) plus insulin (Ins) (0.1 unit/ml); and —□—, insulin (Ins) (0.1 unit/ml) plus dexamethasone phosphate (DEX-P) ( $10^{-6}$  M). Actinomycin D was added 20 min prior to insulin or dexamethasone phosphate. 2-ml samples were taken at 1, 2, 4<sup>3</sup>/<sub>4</sub>, 8, and 12<sup>1</sup>/<sub>2</sub> hr and analyzed as described previously.

which is thought to reflect the translation of previously accumulated TAT message. This increase is not observed if actinomycin D is present during the first incubation. When HTC cells are incubated with insulin and cycloheximide, no increase in TAT activity is observed after removal of the inhibitor and the hormone (Fig. 5). Thus insulin, unlike dexamethasone phosphate, does not appear to cause the accumulation of TAT-specific mRNA, nor the sustained induction of TAT.

**Discussion.** Insulin has been reported to affect diverse functions in animal cells in tissue and organ culture,<sup>17</sup> including effects on membrane function,<sup>18, 19</sup> intermediary metabolism,<sup>19, 20</sup> growth,<sup>5, 6</sup> macromolecular synthesis,<sup>4, 21</sup> and differentiation.<sup>21</sup> In several of these experimental systems, as in HTC cells, serum was found to have similar effects, and in some, insulin could replace an apparent requirement for serum.<sup>4-6</sup>

We have reported here that the addition of 0.1 unit/ml insulin to HTC cells previously induced with dexamethasone phosphate in a serum-free medium causes a rapid two- to threefold increase in the rate of synthesis of TAT, and that this increase is resistant to treatment of the cells with actinomycin D. Furthermore, insulin also stimulates the incorporation of labeled amino acids into protein by about 20 to 30%, and causes a slight shift in ribosome distribution toward polysomal aggregates. These latter effects, however, are at least partly sensitive to inhibition by actinomycin D.

The above effects of insulin are very similar to those evoked by 5% bovine serum, but differ from the latter in the following ways: (1) the insulin effect on TAT synthesis is shorter lived, presumably because of the rapid degradation of the insulin since the readdition of insulin maintains the elevated level of TAT

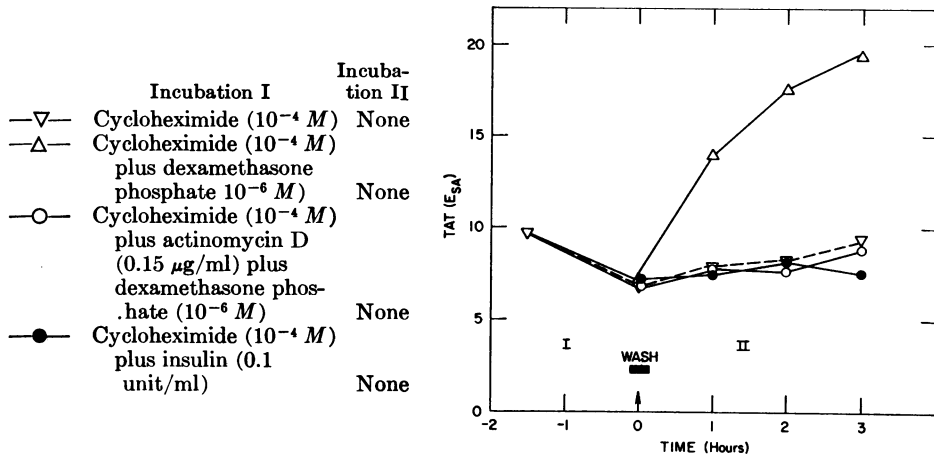


FIG. 5.—Four 10-ml portion of HTC cells in induction medium supplemented with 5% bovine serum were incubated for 90 min with the additions described below. 2-ml samples were removed for enzyme assay, and the cells were centrifuged, washed with fresh serum-supplemented medium without hormones or inhibitors, and resuspended in fresh serum-supplemented medium without hormone or inhibitor. 2-ml samples were taken at 1, 2, and 3 hr incubation and analyzed as described previously.

activity; (2) the insulin effect on TAT synthesis is less sensitive to inhibition by actinomycin D than is the serum effect; (3) insulin has a smaller effect than serum on ribosome distribution and total amino acid incorporation; and (4) most significantly, maximally effective concentrations of insulin and serum have an additive effect on TAT synthesis, which argues that these two factors affect different reactions.

The stimulation of TAT synthesis by insulin also differs from that evoked by glucocorticoids in that insulin does not cause the accumulation of TAT mRNA, nor the sustained induction of TAT. Maximal expression of the insulin effect, however, requires the continued presence of the steroid inducer.

Although the mechanism by which insulin stimulates TAT synthesis is unknown, certain of its aspects may be considered. First, the striking resistance of the insulin stimulation of TAT synthesis to concentrations of actinomycin D which completely inhibit the steroidal induction of TAT suggests that insulin stimulates a step in enzyme synthesis beyond gene transcription. Thus, the insulin effect in HTC cells is quite different from the induction of TAT by insulin in the perfused liver<sup>8</sup> or the intact liver *in vivo*,<sup>7</sup> which is completely inhibited by actinomycin D.

Second, the insulin effect in HTC cells does not involve the adenylyl cyclase-cyclic AMP system, since neither the cyclase nor the cyclic nucleotide are apparently present in HTC cells.<sup>22</sup>

Third, the insulin stimulation of TAT synthesis does not appear to be secondary to an effect on hepatic uptake of glucose or amino acids. Although insulin apparently has a direct effect on hepatic uptake of glucose *in vivo*,<sup>23</sup> and on amino acid uptake in the perfused rat liver,<sup>24</sup> Dickson and Potter<sup>25</sup> have reported that insulin did not affect amino acid uptake by Novikoff and Reuber hepatoma cells in tissue culture. We have observed that the magnitude of the insulin stimulation of TAT synthesis was not proportional to the concentration of either glucose or amino acids in the medium, and that stimulation could even be observed in the absence of either component.<sup>10</sup>

Wool and his colleagues<sup>26</sup> have demonstrated that preparations of ribosomes from skeletal muscle of alloxan-diabetic rats contain fewer polysomes and are less efficient in the *in vitro* synthesis of proteins than ribosomes from normal rats. The administration of insulin *in vivo* to the diabetic animals rapidly corrects these defects by an as-yet-unknown mechanism which requires protein but not RNA synthesis, and is not dependent on the increased cellular uptake of glucose or amino acids. These same workers have localized the protein synthetic defect in diabetic ribosomes to the 60S ribosomal subunit, and have postulated that insulin enhances the synthesis or function of a putative "translation factor" which is associated with the 60S subunit in such a way as to enhance the binding of aminoacyl tRNA.<sup>27</sup> Whether or not these hypotheses are applicable to our observations on the effects of insulin on HTC cells remains to be seen.

We have recently presented a general model for the regulation of induced enzyme synthesis in mammalian cells,<sup>2</sup> in which the formation of specific proteins is inhibited by labile posttranscriptional repressors. The action of the repressor is presumed to be inhibited by enzyme inducers. According to this

scheme, when the inducing steroid is removed from a previously induced HTC cell culture, TAT synthesis stops (see Fig. 2) because translation of the TAT mRNA is inhibited.<sup>2</sup> The observation that insulin can stimulate TAT synthesis in the absence of the inducer (Figs. 2 and 4) suggests that insulin might in some way circumvent the action of the putative repressor. Since insulin and the steroids apparently have different modes of action, it seems unlikely that they influence repressor function in the same way (Figs. 4 and 5).

The relationship between the actions of glucocorticoids, serum, and insulin on the synthesis of TAT is not presently understood, but the finding of three humoral agents which, acting at presumably distinct posttranscriptional sites, control the synthesis of a single protein should provide a useful approach for investigating the regulation of specific gene expression in mammalian cells.

A preliminary account of this work was presented at the meeting of the American Society of Human Genetics in San Francisco, Calif., on October 3, 1969.

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