# AN IN VIVO INSULIN EFFECT ON HEPATOCYTE CHROMATIN DETERMINED BY DNA-RNA HYBRIDIZATION

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SUMMARY: Chromatin was isolated from the parenchymal liver cells of rats that were made insulin deficient with alloxan and from insulin deficient rats that were treated with insulin. RNA was transcribed from each chromatin in vitro by *E.coli* DNA-dependent RNA polymerase. Competitive DNA-RNA hybridizations indicated that there were differences in the RNA transcribed from these two chromatins.

By determining the in vitro rate of RNA synthesis in an incubation mixture containing *E.coli* DNA-dependent RNA polymerase Morgan and Bonner found that the template activity of liver chromatin from insulin deficient rats was 28% less than that from rats treated with insulin (1). Their interpretation of these results was that insulin modulation of enzyme activities in liver cells (2,3) is mediated by derepression of previously repressed genetic material (4). In the experiments described here, DNA-RNA hybridization was used to determine if there are differences in the RNA transcribed from chromatins from the parenchymal liver cells of insulin deficient and insulin treated rats.

## MATERIALS AND METHODS

Male rats of the Sprague-Dawley (Cox) strain were used; the initial body weights were 180-200 grams. Insulin deficient rats were prepared by i.v. injection of 2% alloxan monohydride (Eastman Organic Chemicals), 40 mg per kg of body weight (5,6). Blood glucose determinations were made with the alkaline ferricyanide method as adapted to the Technicon AutoAnalyzer.

The alloxan treated rats had blood glucose levels greater than 300 mg per 100 mls. Three weeks after the alloxan treatment the livers of 5 rats were removed. Another 5 alloxan treated rats was each given a single s.c. injection of 4 units of insulin and 4 units of protamine zinc insulin (Eli Lilly Co.) 24 hours before removal of the livers. The parenchymal liver cells (hepatocytes) were isolated according to the methods of Ingebretsen & Wagle by perfusion with collagenase followed by mild differential centrifugation (7). These hepatocytes were stored frozen at -80° until the time of chromatin preparation.

From the pooled cells of 5 insulin deficient rats, sheared chromatin was prepared by the methods of Bonner, et al (8). Sheared chromatin from the pooled cells of 5 insulin treated rats was prepared by the same methods. Rat DNA was prepared from normal liver chromatin by the procedure of Marmur (9).

The chromatins were transcribed by *E.coli* DNA-dependent RNA polymerase prepared by the method of Chamberlin & Berg (10). This preparation had a DNA dependency of 233x and no RNAse activity. The nucleotides for the RNA substrate were obtained from Calbiochem. [$^{14}$C]ATP, 24 mCi/mmole, was purchased from Schwarz/Mann. Each RNA preparation was synthesized in 10 mls of reaction mixture containing 10 $OD_{260}$ chromatin, 6000 units of DNA-dependent RNA polymerase, 1 mmole tris-HCl (pH 8), 0.1 mmole $MgCl_2$, 0.025 mmole $MnCl_2$, 0.3 mmole of 2-mercapto-ethanol, and 0.02 mmole each of ATP, CTP, UTP, and GTP. To prepare radioisotope labeled RNA, 40 microCi of [$^{14}$C]ATP was added to this incubation mixture. Incubation was at 37° for 15 mins (8,11). The RNA was isolated by phenol extraction (11). From each of the two chromatins, [$^{14}$C]RNA and unlabeled RNA were transcribed.

For the hybridization experiments, rat DNA was denatured in alkali and 0.2 $OD_{260}$ applied to each 11 mm nitrocellulose membrane filter (Schleicher and Schuell B-6) as described by Gillespie and Spiegelman (12). For each concentration of RNA two DNA containing filters sandwiching one blank filter were placed in a small vial with 0.2 ml of hybridization solution containing RNA and 30% formamide (11). After 48 hours at 24°, the filters were removed, washed, treated with RNAse, rewashed, dried, placed in a scintillator and counted for 100 mins.

The RNA transcribed from the chromatins were used in two types of hybridization experiments. The first consisted of adding varying amounts of [$^{14}$C]RNA to DNA filters and determining the % DNA hybridized by the following Equation: (cpm hybridized)/(cpm [$^{14}$C]RNA) x ([RNA])/([DNA]) x 100.

In the second type of experiments, 1 $OD_{260}$ [$^{14}$C]RNA and varying amounts of unlabeled RNA were added to a series of DNA filters. The % hybrid surviving was calculated by using the % DNA hybridized with [$^{14}$C]RNA alone as 100% and determining the relative % DNA hybridized with each concentration of added unlabeled RNA (13,14).

## RESULTS

The [$^{14}$C]RNA transcribed from chromatin from hepatocytes of insulin deficient rats had a specific activity of 13,962 cpm per $OD_{260}$. As shown in Figure 1, with 1.2 $OD_{260}$ of this RNA 15% of the rat DNA on the filter was hybridized, 428 cpm. The [$^{14}$C]RNA transcribed from chromatin from the hepatocytes of insulin treated rats had a specific activity of 12,326 cpm per $OD_{260}$. As shown in Figure 2, with 1.2 $OD_{260}$ of this RNA, 16% of the rat DNA on the filter was hybridized, 395 cpm.

When increasing concentrations of unlabeled RNA transcribed

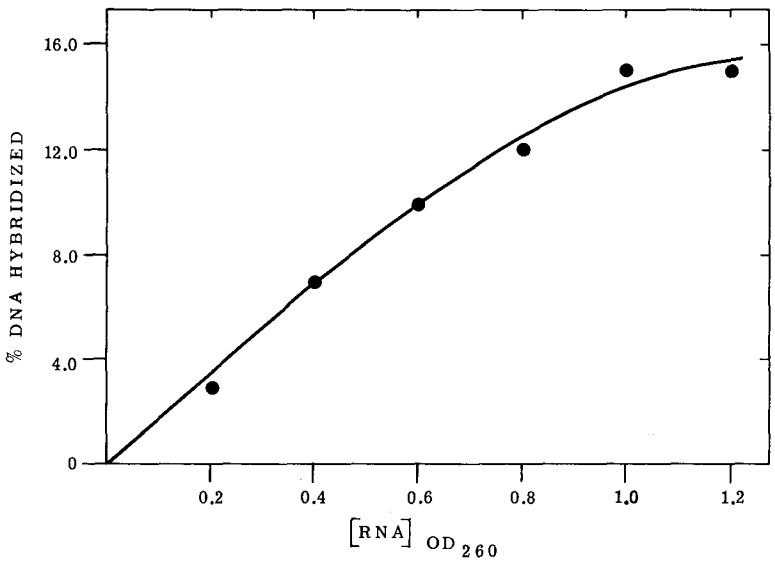


Figure 1. DNA-RNA hybridization. Each filter contained 0.2 $OD_{260}$ normal rat DNA. [$^{14}$C]RNA was transcribed in vitro from hepatocyte chromatin from insulin deficient rats. Each point is the mean of two determinations.

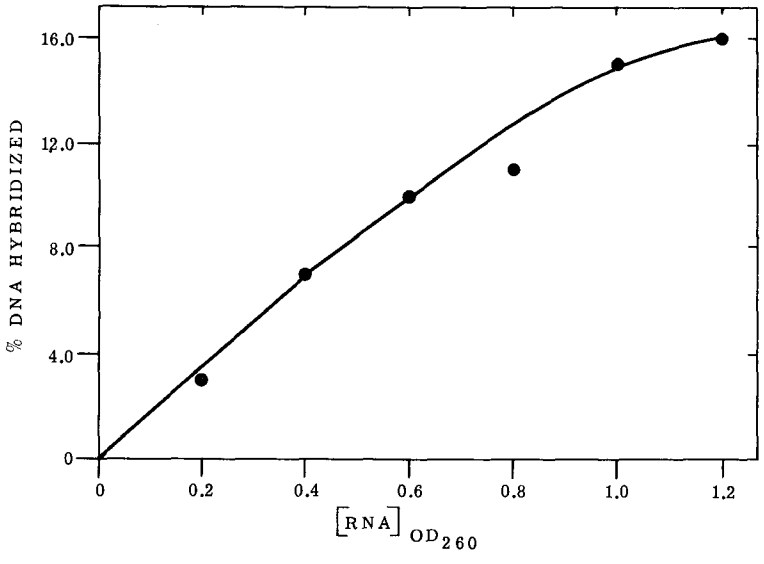


Figure 2. DNA-RNA hybridization. Each filter contained 0.2 $OD_{260}$ normal rat DNA. [$^{14}$C]RNA was transcribed in vitro from hepatocyte chromatin from insulin treated rats. Each point is the mean of two determinations.

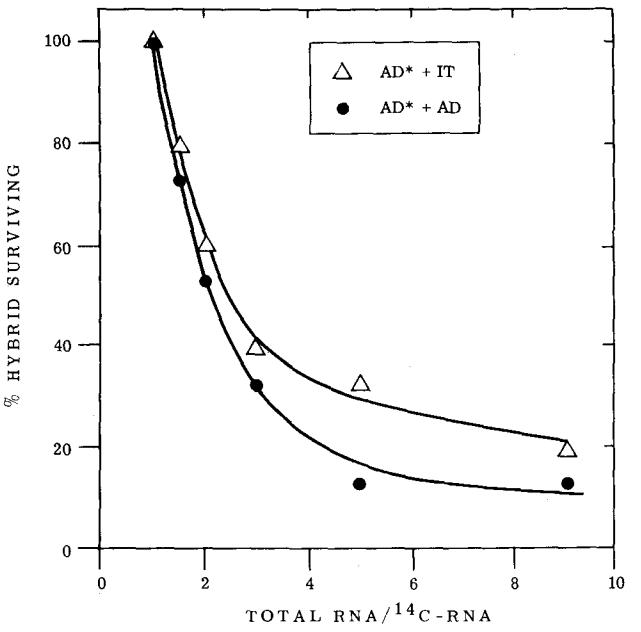


Figure 3. Competitive DNA-RNA hybridization. Each filter contained 0.2 $OD_{260}$ normal rat DNA. Varying amounts of RNA transcribed from hepatocyte chromatin from either insulin deficient (A-D) or insulin treated (I-T) rats were added to 1 $OD_{260}$ [$^{14}$C]RNA transcribed from hepatocyte chromatin from insulin deficient rats (A-D*). Each point is the mean of two determinations.

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from hepatocyte chromatin from insulin deficient rats were added to 1 $OD_{260}$ of [$^{14}$C]RNA transcribed from the same chromatin the % DNA hybridized to the [$^{14}$C]RNA was decreased more than when increasing concentrations of unlabeled RNA transcribed from hepatocyte chromatin from insulin treated rats was added, Figure 3.

Similarly, using [$^{14}$C]RNA transcribed from hepatocyte chromatin from insulin treated rats, unlabeled RNA transcribed from the same chromatin decreased the % DNA hybridized to the [$^{14}$C]RNA to a greater extent than did the unlabeled RNA transcribed from the hepatocyte chromatin from the insulin deficient rats, Figure 4.

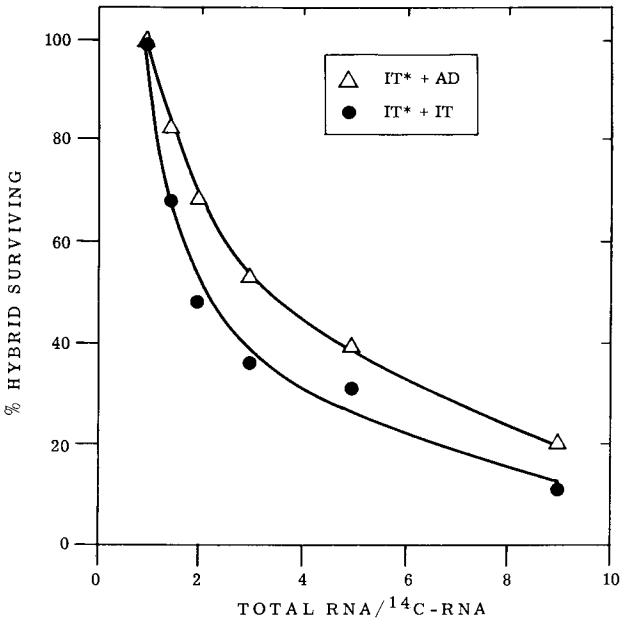


Figure 4. Competitive DNA-RNA hybridization. Each filter contained 0.2 $OD_{260}$ normal rat DNA. Varying amounts of RNA transcribed from hepatocyte chromatin from either insulin treated (I-T) or insulin deficient (A-D) rats were added to 1 $OD_{260}$ [$^{14}$C]RNA transcribed from hepatocyte chromatin from insulin treated rats (I-T*). Each point is the mean of two determinations.

## DISCUSSION

It has been demonstrated that rats treated with 40 mgs alloxan per kg body weight which respond with blood glucose levels in excess of 300 mgs/100 mls are insulin deficient, i.e., with plasma insulin levels less than 20% of normal and pancreatic insulin content less than 10% of normal (6).

Since only 60% of the cells in rat liver are parenchymal cells (hepatocytes), it was important to study the RNA transcribed from hepatocyte chromatin rather than from total liver chromatin (15).

The percent of rat DNA hybridized was approximately the same with [$^{14}$C]RNA transcribed from hepatocyte chromatin from insulin deficient or from insulin treated rats. This would indicate

that the amount of DNA available for transcription was comparable in the two chromatin preparations. However, it does not enable one to determine if different portions of the chromatin DNA are transcribed.

The results of competitive DNA-RNA hybridization shown in Figures 3 and 4 demonstrate that there are differences in the RNA transcribed from these two chromatin preparations.This indicates that the mechanism of action of insulin in liver cells involves the determination of which segments of chromatin DNA are available for transcription (4).

ACKNOWLEDGEMENTS

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