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CIRCADIAN RHYTHM OF COVALENT MODIFICATIONS IN LIVER DNA

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^{32}P-postlabeling analysis recently revealed that in addition to 5-methylcytosine, mammalian DNA contains covalently modified nucleotides of unknown structures and functions termed I-compounds whose levels increase with age. I-compound levels, in addition, depend on species, strain, sex, tissue, and diet and are generally lowered by carcinogen exposure. As shown here, levels of several non-polar I-compounds in liver DNA of untreated male C3H mice were elevated 2 to 8.5 times at 1800 h and 2400 h as compared to 0600 h and 1200 h, while polar I-compounds and persistent carcinogen-DNA adducts induced by safrole were unaffected by time of day. In liver DNA of male F-344 rats 4 non-polar I-compounds and 4 polar I-compounds showed significant circadian rhythm at 2000 h compared to 0800 h. This novel circadian variation of DNA structure implies mechanisms precisely regulating I-compound levels in vivo and may conceivably be linked to diurnal differences of DNA synthesis and gene expression.

Cellular and plasma concentrations of many normal body constituents show circadian variation. Levels of hormones [1], several hepatic microsomal cytochrome P-450 enzymes [2-4], lipogenic enzymes [5], sex hormone receptors [6], transcription and protein synthesis [7,8], and nuclear but not mitochondrial DNA synthesis [9-11] have been reported to show circadian rhythms. Circadian clocks control rhythmicity at different stages of the sequence of events from gene activity to a functional enzyme molecule [8]. We report here for the first time that a special class of structurally modified nucleotides in mammalian DNA termed I-compounds, also, undergoes diurnal changes.

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The abbreviations used are: PEI, polyethyleneimine; TLC, thin-layer chromatography; RAL value, relative adduct labeling value, a minimum estimate of adduct (I-compound) levels in DNA [13].

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I-compounds have been detected by ^{32}P-postlabeling in DNA from various tissues of untreated rodents. These apparently normal constituents of the genome show species, strain, gender, and tissue dependence and gradually accumulate with increasing animal age to levels of several thousand modified nucleotides per genome [12-15]. In order to test the hypothesis that circadian rhythms demonstrated for DNA synthesis and other DNA functions have a structural counterpart in genomic DNA, hepatic I-compound levels were measured by ^{32}P-postlabeling in mice at 0600, 1200, 1800, and 2400 h, and in rats at 0800 and 2000 h.

MATERIALS AND METHODS

Guidelines of the Institutional Review Committee for animal care were followed. Male C3H mice (8-10 wk old, 25-30 g) were purchased from Charles River, Inc. (Wilmington, MA) and housed 5 per cage on a light-dark cycle (light from 0600 to 1800 h) for 2-3 wk prior to and throughout the experiment. Male F-344 rats (10 wk old, 215-230 g) were purchased from Harlan Sprague-Dawley (Houston, TX), housed 3-4 per cage, and acclimatized similarly to mice. Rodent chow (Teklad LM 485) and water were available ad libitum to all the animals.

In Experiment 1, 5 mice each were sacrificed at 0600, 1200, 1800, and 2400 h. Livers were quickly removed, cleaned, minced, frozen in dry ice, and stored at -80°C until DNA isolation [16]. DNA from individual animals was analyzed for covalent modifications by the nuclease P1-enhanced deoxyribonucleoside 3', 5'-bisphosphate version of the ^{32}P-postlabeling assay [17] employing published conditions for I-compound separation [18].

In Experiment 2, liver DNA from groups of 5 mice each was examined at 0600 and 1800 h on day 1, and 0600 h on day 2.

In Experiment 3, a single dose of the hepatocarcinogen safrole (20 μg/mouse, dissolved in 100 μl trioctanoin) was given i.p. to 16 mice at 1000-1015 h. Eight control mice each received 100 μl solvent. Three weeks later 4 safrole-treated and 2 solvent-treated mice each were killed at 0600, 1200, 1800, and 2400 h and their individual liver DNA's examined.

In Experiment 4, 3 male F-344 rats each were sacrificed at 0800 and 2000 h, livers were collected, and DNA from individual animals was analyzed as in Experiment 1, using modified chromatographic conditions. Briefly, the D1 chromatogram was cut into L and C portions [18] containing non-polar and polar ^{32}P-labeled I-compound fractions, respectively, as previously described [15]. Non-polar I-compounds from L cut-outs [18] were resolved by PEI-cellulose TLC employing 2.76 M lithium formate, 4.88 M urea, pH 3.35 in the first dimension, followed by 0.49 M NaH_2PO_4, 4.9 M urea, pH 6.4 in the second dimension. Polar I-compounds from the C cut-outs [18] were resolved with 3.05 M lithium formate, 5.4 M urea, pH 3.35, followed by 0.21 M NaH_2PO_4, 2.1 M urea, pH 6.4.

RESULTS AND DISCUSSION

In Experiment 1 intensities (Fig. 1A) and levels (Fig. 1B) of ^{32}P-postlabeled non-polar I-compounds 2, 3, and 4 were significantly (t-test, $P < 0.05$) higher at 1800 and 2400 h as compared to 0600 and 1200 h, while spot 5 showed an opposite trend ($P < 0.001$). Spot 1 was diminished significantly at 0600 h only ($P < 0.05$). RAL values of spots 2-5 at 1200 h did not differ from those at 0600 h. Levels of I-compounds 1, 2, 4, and 5 did

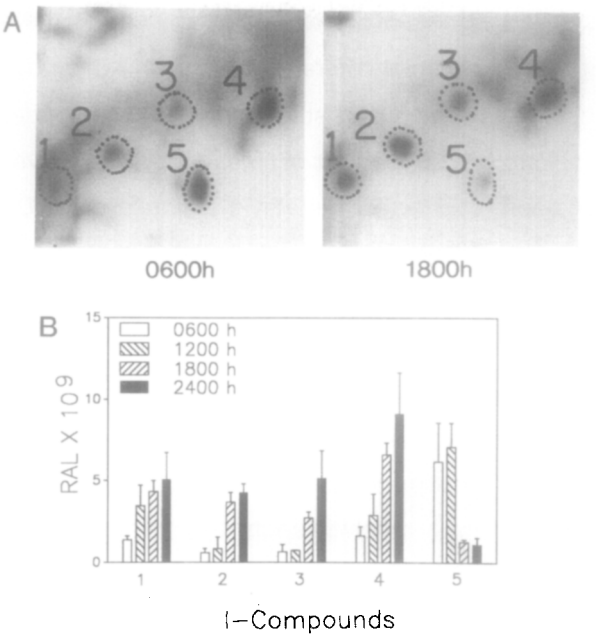

Fig. 1. Results of Experiment 1. (**A**) Autoradiograms of 2-dimensional PEI-cellulose TLC maps of ^{32}P-labeled non-polar I-compounds isolated from male C3H mouse liver DNA at 0600 and 1800 h. Film exposure was at -80°C for 16 h using Kodak XAR-5 film and Du Pont Lightning Plus intensifying screens. DNA from five individual livers was analyzed by nuclease P1-enhanced ^{32}P-postlabeling [17, 18]. ^{32}P-labeled fractions were identified as I-compounds by TLC comparison with previously identified mouse liver I-compounds [22, 24]. Spots 1-5 were excised, counted, and relative adduct labeling [RAL X 10^9 (± SD)] values, representing a minimum estimate of the number of modified nucleotides in 10^9 DNA nucleotides, calculated [17]. (**B**) RAL X 10^9 (± SD) values of I-compounds 1-5 from C3H male mouse liver DNA.

not vary significantly between 1800 and 2400 h ($P < 0.10$), but the RAL value of spot 3 was significantly higher at 2400 h than at 1800 h ($P < 0.01$). Polar I-compound levels of the 8-10 wk old mice were low and not significantly affected by time of day. Experiment 2, which included a 0600 h time point on day 2, gave comparable results: I-compound levels were significantly higher (spots 1-4) or lower (spot 5) at 1800 h on day 1 as compared to 0600 h on days 1 or 2, and there were no significant differences between the latter groups (data not shown).

In order to determine whether circadian variation is a unique property of I-compounds or is shared by other covalent DNA modifications such as carcinogen-DNA adducts, levels of both I-compounds and persistent adducts induced by the hepatocarcinogen safrole in mouse liver DNA were examined in Experiment 3. Safrole is known to give 2 major chemically identified adducts by ^{32}P-postlabeling [19]. Total safrole adduct levels corresponded to 17.4 ± 1.1, 17.4 ± 0.7, 18.3 ± 1.5, and 20.7 ± 1.9 at 0600, 1200, 1800, and 2400 h, respectively. Only I-compounds but neither individual nor total

safrole adducts showed significant ($P < 0.05$) differences at the 4 time points. These observations suggest fundamental differences between I-compounds and carcinogen-DNA adducts in that only the latter persist in tissue DNA without detectable change over short (this report) or long [19, 20] periods of time. A literature survey showed that diurnal variations have not been reported for 5-methylcytosine levels in DNA.

The circadian rhythm of I-compound levels in male F-344 rat liver DNA was examined at 0800 and 2000 h. Representative autoradiograms for 0800 h and data for 0800 h and 2000 h time points are shown in Fig. 2. Levels of 4 non-polar I-compounds, i.e. spots 1, 2, 5, and 6 were significantly different at the 2 time points (Fig. 2C), with spots 5 and 6 being undetectable at 2000 h. The level of spot 1 increased ($P < 0.05$) and that of spot 2 decreased ($P < 0.05$) significantly at 2000 h compared to 0800 h. Spot 4, which represented a cluster of partially resolved spots (Fig. 2A) and thus was taken as a single area, was unaffected by time of day. This was also the case for spot 3. Levels of 4 polar I-compounds, i.e. spots 8 ($P < 0.05$), 9 ($=0.05$), 11 (<0.05), and 13 (<0.05) were

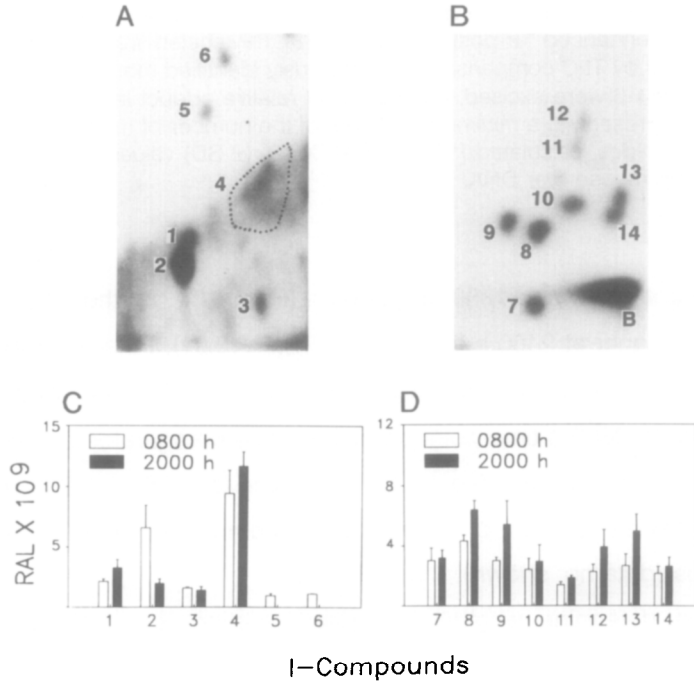

Fig. 2. Results of Experiment 4. Autoradiograms of 2-dimensional PEI-cellulose TLC maps of **(A)** non-polar and **(B)** polar I-compounds isolated from male F-344 rat liver DNA at 0800 h. Film exposure was at -80°C for 16 h using Kodak XAR-5 film and Du Pont Lightning Plus intensifying screens. DNA from 3 individual rat livers was analyzed by ^{32}P-postlabeling [17]. Spots 1-6 (L maps) and 7-14 (C maps) were excised, counted, and relative adduct labeling [RAL x 10^9 (± SD)] values calculated as in Fig. 1. **(C)** RAL x 10^9 (± SD) values of non-polar I-compounds from L maps, and **(D)** polar I-compounds from C maps. Spot B (panel B) denotes radioactive background material.

significantly increased at 2000 h compared to 0800 h. Spots 7, 10, 12, and 14 did not show significant time-dependent differences. In contrast to most non-polar I-compounds (Fig. 2C), levels of all the polar I-compounds tended to be elevated at 2000 h (Fig. 2D).

In conclusion, a number of I-compounds in mouse and rat liver DNA showed significant circadian variation, as determined by ^{32}P-postlabeling. While the low amounts of individual I-compounds in tissue DNA have thus far prevented their structural characterization, several lines of evidence suggest functional roles of these DNA modifications [12-15]. On the basis of observations in aging [14] as well as carcinogen-exposed [21-23] animals, it appears that I-compound levels in tissue DNA are controlled by as yet poorly characterized mechanisms which determine their formation and removal. Experimental evidence shows that both genetic and environmental factors contribute to these processes [13-15, 24], and that drug-metabolizing (cytochrome P450) enzymes are involved [25, 26]. The present results support the hypothesis that control of I-compound levels may be essential for mammalian DNA function.

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